1951

# Purification and properties of bacterial pyrimidine nucleosidase

Lynn Merton Paege
*Iowa State College*

# NOTE TO USERS

# PURIFICATION AND PROPERTIES OF BACTERIAL PYRIMIDINE NUCLEOSIDASE

by

Lynn Merton Paege

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Physiological Bacteriology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State College
1951

TABLE OF CONTENTS

# INTRODUCTION

One of the most important developments of physiological bacteriology in recent years is the increasing awareness of the significance of nucleic acids. The nucleic acid content of cells often has been underestimated in the past. In bacteria it occurs in concentrations which, except for some of the acid-fast bacteria, are larger than those of lipoid material. With the exception of organisms which consist of large amounts of carbohydrate (located principally in the capsular material) nucleic acids occur in concentrations higher than those of carbohydrate. Protein is the only major fraction which always exceeds the nucleic acid content in quantity. Although nucleic acid is second to protein in amount in bacteria, the significance of its function is as undisputed as that of protein.

The importance of nucleic acids in the genetic make-up of cells is widely appreciated. Mutations, caused by irradiations for example, have established this point beyond a doubt. The bacteriologist has a very impressive example in the pneumococcus type transformation principle, which at least for the transformation IIR to IIIS, has been found to be a desoxyribose nucleic acid. Recently the concept of the multiple nature of ribose nucleic acid and desoxyribose

nucleic acid has been proposed and although the possibilities of variety are not as large as those of protein, there is no difficulty in assuming an individual nucleic acid for each gene.

Unfortunately we do not have a single instance in which we might formulate the participation of nucleic acid in a biochemical reaction in a fashion such as that by which we can now explain the participation and mode of action of many coenzymes. While the developments in the coenzyme nucleotide field constitute one of the most spectacular phases of biochemical development, an analogous accomplishment with polynucleotides or nucleoproteins is yet to come.

The enzymology of nucleic acids is restricted to date to the study of the enzymes concerned with the assimilation and dissimilation of nucleic acids. These enzymes may be subdivided into several groups as follows:

A. Enzymes concerned with the formation and breakdown of the structural units of nucleic acids. These may be subdivided into:

   1. Enzymes of purine synthesis.
   2. Enzymes concerned with the formation of pyrimidines.
   3. Enzymes of ribose and desoxyribose metabolism.
   4. Enzymes regulating the supply of phosphoric acid for nucleic acid synthesis by splitting

phosphoric acid from organic phosphoric acid esters or by regulation of the utilization or accumulation of reserves in the form of metaphosphate.

B. Enzymes concerned with the combination of structural units to nucleic acids and nucleoproteins. These may be grouped as follows:

1. Purine nucleosidases (purine nucleoside phosphorylases) combining base and carbohydrate to nucleosides.
2. Pyrimidine nucleosidases combining pyrimidine bases with ribose or desoxyribose.
3. Nucleotidases which interconvert nucleosides and mononucleotides by phosphorylation or dephosphorylation.
4. Nucleases, a group of enzymes which are ill-defined and which in some instances comprise ribonuclease, desoxyribonuclease, phosphodiesterase and possibly other enzymes.

A perusal of recent reviews on this subject shows that our knowledge is indeed limited with respect to almost every phase of nucleic acid enzymology enumerated above. It is evident that any investigation of individual enzymes concerned will contribute to the development of the entire perspective.

The study presented here deals mainly with bacterial pyrimidine nucleosidase. At the outset of the work there was only scant information on this group of enzymes from investigations with animal tissues. Bacterial pyrimidine nucleoside metabolism had remained untouched. While this work was in progress and after the first report had been published other laboratories joined in the development. The extent of the concerted efforts reflects the significance of the problem.

## REVIEW OF LITERATURE

Previous to 1911, enzymes concerned with the metabolism of nucleic acids were grouped under the rather inclusive title "nucleases", a term first used by Iwanoff (1903). According to Jones (1911a, p. 129) "nuclease" was described as a "ferment almost or quite universally present in animal glands, through whose agency nucleic acid is decomposed with the liberation of purine bases which thus become directly precipitable by the reagents commonly employed for this purpose". In view of the investigations of Jones and his co-workers, those of others and their own experimentations, Levene and Medigreceanu (1911c) suggested that the disintegration of nucleic acids to the purine bases is a result of the graded action of several enzymes. They proposed the following nomenclature according to the activity which the enzymes performed: (1) nucleinases, enzymes causing the dissolution of the nucleic acid into nucleotides, (2) nucleotidases, enzymes which act upon nucleotides to form phosphoric acid and a carbohydrate-base complex and (3) nucleosidases, enzymes which cleave nucleosides into base and ribose. Thus the naming as well as the discovery of nucleosidase is attributed to Levene.

Levene and Medigreceanu (1911a,b,c) found the enzyme

nucleosidase widely distributed in all animal organs, with the best preparations coming from bovine pancreas. The method of preparation of the crude enzyme consisted of grinding the organ with quartz sand, expression in a Buchner press, and autolysis. Nucleosides were hydrolyzed to free base and ribose by the enzyme. It was observed at this time that pyrimidine compounds were acted upon much less readily than were their purine counterparts. These authors also reported (1911b) that gastric and pancreatic juices did not metabolize nucleosides. Komita (1938b) however, was able to show that nucleosides were split by these juices if inorganic phosphate were provided for the reaction. He offered no interpretation for the effect; apparently its significance was overlooked.

Contemporary with the work of Levene and co-workers was that of Jones (1911b). He was more concerned with nucleotidases and enzymes then known as desamidases prepared from pig pancreas. It is significant to note that he suggested that purine compounds may be "desamidized" before the nucleoside is cleaved to its final components. Jones also suggested that the enzymes of pig pancreas were able to attack nucleic acid at two different points. In one case free purine bases were formed, in the other two purine nucleosides (guanosine and adenosine) and phosphoric acid were formed.

That uridine was refractory to hydrolysis of dilute mineral acid was shown by Levene and LaForge (1910, 1912). It was also reported that hydrogenated uridine, (dihydrouridine), is easily split under these conditions, as are the purine nucleosides. It was thought that nucleosidase might behave similarly; however, the enzyme was inactive against dihydrouridine (Levene and LaForge, 1913).

Little more was done with respect to nucleosidase until 1924 when a series of papers appeared by Levene and co-workers (Levene, Yamagawa and Weber, 1924; Levene and Weber, 1924a,b). The enzyme was extracted from minced organs with phosphate buffer and autolyzed. Purification was effected by adsorption on cholesterol, stearic acid, kaolin, aluminum hydroxide, or colloidal iron. Kaolin adsorbed the enzyme so strongly that it was difficult to re-extract. Aluminum hydroxide failed to adsorb the enzyme at the pH used. Colloidal iron was most satisfactory and was used as an adsorbent for purification by fractional adsorption. The enzyme adheres very tenaciously, but was extracted from the colloidal iron with disodium phosphate or sodium hydroxide at pH 8.76. The neutralized extracts were then concentrated under reduced pressure and the enzyme was precipitated with acetone and dried in a vacuum disiccator. The purified enzyme exhibited the following characteristics: (1) it was not active against adenine "hexosides" or nucleic

acid, (2) when adenosine was used as a substrate, the products of the reaction, ribose and adenine, exerted a retarding influence on the reaction, (3) the optimum pH for activity was 7.5, and (4) the optimum temperature for activity was 37°C.

Later various methods for the purification of nucleosidase were investigated by von Euler and Brunius (1927). The enzyme was extracted from ground swine kidney. Attempts to purify it by adsorption on colloidal iron were largely unsuccessful. The enzyme could not be adsorbed on kaolin from acid, neutral or alkaline solutions. Alumina adsorbed it quantitatively at pH 4.91; it was eluted with $Na_2HPO_4$ - NaOH solution at pH 9. However, no purification resulted from this method; the impurities were eluted along with the nucleosidase. The method recommended for the preparation of active enzyme in good yield was fractional precipitation with acetone. With regard to the activity of the enzyme, it was reported that with adenosine as substrate, the equilibrium was established at 73 per cent hydrolysis. A marked inhibition of the reaction by nucleic acid or uric acid was observed.

As indicated earlier, Levene and Medigreceanu (1911c) reported that the nucleases capable of the metabolism of purine nucleosides were ineffective in the hydrolysis of pyrimidine nucleosides. The enzymology of pyrimidine

nucleosides did not progress until 1929 when Deutsch and Laser (1929) obtained a nucleosidase which was active against pyrimidine nucleosides and much less active toward adenosine. The enzyme was prepared from a juice obtained by subjecting bone marrow to pressure in a Buchner press. The juice was diluted with water and allowed to autolyze. Purification of the enzyme was difficult since it was sensitive to alkali and methyl acetate. The activity was increased by precipitation of the proteins at their isoelectric points. Aside from the preferential hydrolysis of pyrimidine nucleosides by the enzyme, it differed from purine nucleosidase in the following respects: (1) the pH for optimum activity was found to be 6.5 whereas that of purine nucleosidase was 7.5, (2) pyrimidine nucleosidase was sensitive to alkali in contrast to purine nucleosidase which was found stable at pH 10.08 for as long as two hours (Levene and Weber, 1924b), (3) pyrimidine nucleosidase was distributed differently from purine nucleosidase in different tissues. These facts and the fact that the ratio of the two enzymes changed during purification made it quite clear that pyrimidine nucleosidase is an enzyme separate and distinct from purine nucleosidase.

The specificity of purine nucleosidase was further established in 1931 by Levene and Dmochowski (1931). The crude enzyme preparation of Levene and Weber (1924) contain-

ed both nucleosidase and nucleotidase. After purification of the enzyme by adsorption and elution from alumina and colloidal iron, the enzyme preparation showed no activity toward adenylic and guanylic acids. It was thus established that nucleosidase is incapable of separating the purine base from the nucleotide.

Somewhat later, Schmidt (1932) showed that an aqueous extract of rabbit liver formed ribose-phosphoric acid ester from guanylic acid. Deamination was accompanied by simultaneous liberation of a purine base. The ribose-phosphoric acid ester was isolated as the barium salt. It was felt that these results did not conflict with those of Levene and Dmochowski (1931) since the liberation of ammonia must be due to the combined action of nucleotidase, nucleosidase and guanase.

One of the more complete studies of nucleosides and their enzymology was performed by Dixon and Lemberg (1934). Experiments were carried out with an unpurified xanthine oxidase of milk which contained nucleosidase. The results led these authors to believe that nucleosidases were quite specific, contrary to popular opinion at that time. They pointed out that if nucleosidases were relatively non-specific ribosidases, they would be expected to hydrolyze other ribosides. Yet as early as 1912, Levene, Jacobs and Medigreceanu (1912) were able to show that nucleosidase

could hydrolyze neither α- nor β-methyl-d-riboside. Thus the conclusion must be drawn that nucleosidase shows specificity toward the non-ribose part of the molecule. According to these authors, added evidence that nucleosidase is not a ribosidase, is the special pyrimidine nucleosidase of Deutsch and Laser (1929) described above. This also indicates the specificity of the enzyme for the non-carbohydrate part of the molecule. The nucleosidase of Dixon and Lemberg was extremely specific for the purine part of the molecule. The enzyme was active against inosine converting it to hypoxanthine, but it showed no activity toward xanthosine. In the presence of intestinal phosphatase, inosinic and adenylic acids behave the same as inosine and adenosine. It was therefore suggested that the term purine nucleosidase should imply activity toward guanosine, desoxyguanosine, inosine and desoxyinosine.

The next effort to study nucleosidase activity was that of Klein (1935). Nucleosidase was prepared from extracts of frozen spleen, lung, liver or heart tissue. The enzyme was purified by adsorption and elution from alumina. Klein (1935) reported the enzyme to show the following characteristics. (1) Arsenate and phosphate act as activators. The addition of phosphate restores the activity lost through dialysis. (2) The enzyme is specific for purine nucleosides. It has no effect upon pyrimidine nucleosides or nucleotides,

nor do pyrimidine nucleosides or nucleotides effect the reaction. (3) Guanine and hypoxanthine are strong inhibitors of the reaction, adenine is less inhibitory and desoxyribose is but slightly inhibitory. Both ribonucleosides and desoxyribonucleosides are attacked, therefore purine ribose nucleosidase and purine desoxyribose nucleosidase is one and the same enzyme. Pyrimidine nucleosidase is a separate and distinct enzyme.

Lutwak-Mann (1936) was the first one to use bacteria to study the metabolism of adenine compounds. Using whole cells of Bacterium coli (E. coli) it was found that the bacteria were able to decompose the following compounds: adenosine triphosphate, muscle and yeast nucleic acids, adenosine, and adenine. Hypoxanthine was the product of the reaction. The ribose liberated was metabolized by the organisms, since none was found in the reaction mixture. Guanylic acid was dephosphorylated, and in the presence of phosphate the bacteria were able to deaminate guanosine.

This work was followed by that of Stephenson and Trim (1938). Muscle adenylic acid was dephosphorylated and deaminated. The experiments seemed to indicate that dephosphorylation preceded deamination. Adenosine and inosine were both metabolized with the production of ribose, no trace of which was found; it was completely metabolized by

the Bact. coli cells used.

While working with a crude kidney enzyme preparation containing nucleosidase, Inagaki (1940) observed that guanine desoxyriboside was hydrolyzed somewhat faster than guanine riboside, and that thymine desoxyriboside was hydrolyzed but slightly. The following suppositions concerning the enzymes involved were submitted. (1) Special enzymes exist for the hydrolysis of ribonucleosides and desoxyribonucleosides, since the sugar determines the hydrolyzability of certain glycosides. (2) One and the same enzyme may hydrolyze linkages at different rates. As yet no one has been able to establish which of the postulates is correct.

Dixon and Lemberg (1934) pointed out that the specificity of purine nucleosidase rested largely in the non-carbohydrate portion of the molecule. However, Schaedel, Waldvogel and Schlenk (1947) observed that their nucleosidase preparation could split adenosine, but would cleave neither isoguanine, adenosine thiomethylpentoside, nor xanthine thiomethylpentoside. This emphasizes the fact that the carbohydrate fraction of the molecule also confers specificity to the enzyme.

Evidence for the formation of ribose-1-phosphate as one of the products of the enzymic splitting of nucleosides was presented by Kalckar (1945a,b). Nucleosidase, prepared from

extracts of rat liver and purified by fractional precipitation with ammonium sulfate, catalyzes the following reaction:

Ribose-1-purine + phosphate $\rightleftharpoons$ ribose-1-phosphate + purine

Ribose-1-phosphate was isolated as the barium salt. Phosphate or arsenate was required in order that splitting occur, and for each mole of purine liberated, one mole of phosphate was bound in an acid-labile organic linkage. Preliminary evidence for the reversal of the reaction was given. Inorganic phosphate was liberated when ribose-1-phosphate and hypoxanthine was incubated with the enzyme. This work was extended in 1947 (Kalckar, 1947c,d). Inosine and guanosine were synthesized enzymatically by the incubation of hypoxanthine or guanine with ribose-1-phosphate in the presence of nucleoside phosphorylase. The position of the equilibrium favors riboside formation. Ribose, ribose and phosphate, or glucose-1-phosphate could not be substituted for ribose-1-phosphate in the reaction. It was suggested that the reaction of nucleosidase was analogous to that of Cori's polysaccharide phosphorylase in the formation of glucosidic linked hexose chains (Cori, 1943) and to the disaccharide phosphorylase of Doudoroff for the synthesis of sucrose (Doudoroff, 1943); consequently, nucleoside phosphorylase is the proper name for the enzyme.

Friedkin, et al. (Friedkin, Kalckar and Hoff-Jørgensen, 1949; Friedkin and Kalckar, 1950; Friedkin, 1950; Hoff-Jørgensen, Friedkin, and Kalckar, 1950), using the nucleoside phosphorylase of Kalckar (1947), were able to show the formation of desoxyribose-1-phosphate. Both inosine and hypoxanthine desoxyribosides were split by the enzyme. Inorganic phosphorus was required for the reaction. Hypoxanthine desoxyriboside was synthesized when hypoxanthine and desoxyribose-1-phosphate were incubated in the presence of nucleoside phosphorylase. The equilibrium favored synthesis of the desoxyriboside rather than splitting.

Manson and Lampen (1947, 1951a,b) have also studied the metabolism of desoxyribosides using cells of E. coli and the nucleoside phosphorylase of Kalckar (1947). E. coli contains enzymes which cause desoxyribose nucleosides to undergo phosphorolysis to free base and desoxypentose phosphate. The desoxypentose does not accumulate, but is degraded by the cells. The purine nucleoside phosphorylase of rat liver also cleaves hypoxanthine desoxyriboside with the formation of desoxyribose-1-phosphate. In addition the enzyme preparation contains an enzyme phosphodesoxyribomutase, which converts desoxyribose-1-phosphate to desoxyribose-5-phosphate.

Wang (1950) and Wang, et al. (1950) demonstrated the presence of an enzyme system in cell-free extracts of E. coli

which would deaminate cytidine and cytosine desoxyriboside. Adenosine, adenine, cytosine, isocytosine guanine and guanosine are also deaminated by the extracts.

Cells of Lactobacillus pentosus have been shown to contain nucleosidases active against adenosine, cytidine and xanthosine by Wang and Lampen (1951). Corresponding desoxyribosides were split slowly if at all. Attempts to purify and extract of the cells of Lactobacillus pentosus by fractionation with ammonium sulfate was unsuccessful.

Recently, Cardini, et al. (1950) presented evidence for the existence of an enzyme in rat liver which they called uridine phosphorylase. In the presence of inorganic phosphate, the enzyme converted uridine to uracil and ribose-1-phosphate. Uridylic acid was also split, but neither cytidine nor cytidylic acid was attacked.

Carter (1951) presented evidence for a non-phosphorolytic uridine nucleosidase, purified from yeast. The nucleosidases previously described have been phosphorolytic in nature. This one is characterized by the unusual ability to hydrolyze uridine in the absence of phosphate or arsenate. The enzyme is specific for uridine; purine nucleosides, cytidine and thymidine are not attacked. The existence of this hydrolytic nucleosidase may indicate an isolated or alternative pathway of riboside metabolism.

Two unusual enzyme systems related to the nucleosidases

must also be mentioned. The one, nucleotide-N-ribosidase, presented a scheme for the enzymic degradation of nucleotides foreign to the one now generally accepted. The other, ribonucleic acid phosphorylase, has been withdrawn by its author; the experiments could not be repeated.

Early work on the enzymic degradation of nucleotides had indicated that nucleotidase action must precede action of nucleosidase, i.e. the nucleotide must be dephosphorylated before the glucosidic bond can be ruptured (Jones, 1920, p. 93; Levene and Bass, 1931, p. 311). Beginning in 1936, a series of papers (Ishikawa and Komita, 1936; Komita, 1937, 1938a,b) appeared in which it was suggested that enzymic hydrolysis of nucleotides may occur via two methods. (1) Primary detachment of the ester linkage followed by hydrolysis of the glucosidic bond (which corresponds to the generally accepted scheme). (2) Primary hydrolysis of the glucosidic linkage followed by decomposition of the ribose phosphoric acid ester. Evidence was submitted for the splitting of guanylic acid and xanthylic acid, by an enzyme purified from dog pancreas, according to the following reaction:

Guanosine-3'-phosphate → guanine + ribose-3-phosphate

Upon prolonged incubation nucleotides were split at the glucosidic linkage. Hydrolysis of this linkage took place

without dephosphorylation or deamination; there was no increase in inorganic phosphorous or ammonia. The ribose-phosphoric acid isolated from the reaction was not well characterized. It was shown however, that ribose-3-phosphoric acid and ribose-5-phosphoric acid markedly influence the reaction. Thus the exact position of the phosphoric acid of the ester remained open to question. The enzyme, which was called nucleotidase-N-ribosidase does not require phosphate or arsenate for activity as does purine nucleosidase. During purification the activity of nucleosidase and nucleotide-N-ribosidase increased in a parallel fashion. Both enzymes were adsorbed on alumina C-gamma but only nucleosidase could be eluted from the adsorbent. It was possible to separate the two enzymes by selective elution from colloidal iron. Nucleotidase-N-ribosidase is strongly influenced by the products of the reaction, namely guanine and the ribose phosphoric acid esters mentioned above. Schlenk (1949) suggested that a new interpretation of the results was possible. The nucleoside was dephosphorylated and the resulting ribose-1-phosphate stabilized to ribose-5-phosphate (Schlenk and Waldvogel, 1946, 1947). Until it is possible to isolate ribose-3-phosphate, the existence of Komita's nucleotide-N-ribosidase cannot be proved.

Colowick and Price (1945, 1946) suggested the presence of an enzyme in aqueous extracts of rat muscle which in the

presence of inorganis phosphate, liberates guanine from ribonucleic acid. The enzyme, ribonucleic acid phosphorylase, catalyzed the reaction between ribonucleic acid and inorganic phosphate in which the products were free guanine and a derivative of ribonucleic acid ("P-ribonucleic acid") in which guanine groups had been replaced by phosphate groups. Specificity for ribonucleic acid was claimed. However, Colowick (1947) reported that the experiments could not be repeated, and that the findings described above appear to be erroneous.

The enzyme of pyrimidine nucleoside metabolism are important not only with respect to the anabolism and catabolism of nucleic acids, but in another connection also. A coenzyme containing uridine was reported recently by Cardini, et al. (1950) and Caputto, et al. (1950). This coenzyme for the enzyme waldenase, which converts galactose-1-phosphate to glucose-1-phosphate, was isolated from animal tissue and yeast. The yeast may or may not be adapted to galactose; therefore, other functions of the coenzyme are probable. The coenzyme was called UDPG, uridine-diphosphate-glucose; and tentatively, the following formulation has been assigned: uridine-5-pyrophosphate-glucose, with the glucose attached at carbon atom number one.

# METHODS AND MATERIALS

## Organisms.

The organisms employed in the following investigation were Escherichia coli E-26 (Iowa State College laboratory culture), Aerobacter aerogenes 2-A-8 (Iowa State College laboratory culture) and Micrococcus lysodeikticus 3-A-1 (Iowa State College laboratory culture.) Cultures were maintained on nutrient agar slants and transferred once a month.

In order to obtain a good yield of various nucleolytic enzymes, the medium must be low in carbohydrate and high in nitrogen; a low pH at the end of the culture period must be avoided. The following medium satisfied these requirements and was used for the culture of E. coli and A. aerogenes. It was composed of 1.5% peptonized milk, 0.75% $K_2HPO_4 \cdot 3H_2O$, 10% tap water, made to volume with distilled water. The phosphate was dissolved in tap water and the pH was adjusted to neutrality with concentrated $H_3PO_4$. This solution was sterilized separately and added aseptically to the remainder of the medium prior to inoculation. The solutions were sterilized by autoclaving at 15 pounds pressure for 25 minutes. E. coli or A. aerogenes was inoculated into a 100 ml. portion of the medium. After 12 hours incubation at

37°C. the 100 ml. volume was inoculated into a liter portion of the medium and incubated for 12 hours at 37°C. This was then inoculated into the remaining 9 liters of the medium. The incubation was carried out in a 12 liter Florence flask for 18-20 hours at 30°C. under vigorous aeration.

The cells were harvested with a Sharples centrifuge. The cell paste was washed twice with 0.05 M phosphate buffer, pH 7.0, and sedimented in a high speed angle centrifuge after each washing. Whenever the presence of phosphate ion was undesirable in the experiments contemplated, the cells were washed twice with distilled water. Cells were used within 24 hours after harvesting and were refrigerated until used.

M. lysodeikticus was grown in Roux flasks on a solid medium containing 1% glucose, 0.3% yeast extract, 0.3% peptone, 2% agar, 10% tap water, made to volume with distilled water. Approximately 200 ml. of medium was added to each Roux flask. The flasks and contents were sterilized by autoclaving at 15 pounds pressure for 15 minutes. Cultures for the inoculum were prepared on agar slants. The slants were incubated at 30°C. for 72 hours. The cells were washed from the slants with sterile distilled water. The washings were transferred aseptically into a sterile nasal atomizer. The agar surfaces in the Roux flasks were then sprayed with

the inoculum. After 3 days incubation at 30°C. the cells were harvested by washing the surface with distilled water. The washings were filtered through glass wool to remove agar particles which might have been dislodged. After centrifugation the cell paste was washed once with distilled water.

## Bacterial Enzyme Preparations.

### Whole cells.

Convenient amounts of the washed cell paste were weighed and suspended in water just prior to use.

### Acetone-treated cells.

In early experiments the cells were treated with acetone in the following manner. The paste of fresh cells was shaken vigorously with 7 parts of ice-cold acetone for 5 minutes. The cells were removed by filtration and suspended in 14 parts acetone and shaken for 10 minutes. The second acetone treatment was repeated. After filtration, traces of adherent acetone were removed by evacuation in a vacuum desiccator.

Later the procedure was modified. The paste of fresh cells was suspended in 7.5 volumes ice-cold 0.05 M phos-

phate buffer, pH 7.0. 35 volumes ice-cold acetone were added gradually. The mixture was stirred vigorously with an air-driven stirring motor for 5 minutes. The cells were filtered and suspended in 25 volumes ice-cold acetone and stirred vigorously for a second 5 minute period. After filtration the cells were placed in a vacuum desiccator to remove adhering acetone. Cells prepared in this manner were more easily suspended in water and showed greater nucleosidase activity than cells treated in the manner described above.

Lyophilized cells.

Lyophilization was carried out by suspending fresh cells in sufficient distilled water to make a slurry. The suspension was then transferred to a round-bottomed flask and rapidly frozen in an acetone-dry ice bath. The cells were dehydrated in the frozen state under vacuum.

Acetone-treated and lyophilized cells were stored in a desiccator in the refrigerator. Nucleosidase activity remains constant for several months when the cells are preserved in this manner.

Cell-free extracts.

Two methods were used for the preparation of cell-free

extracts.

In one method, extracts were prepared by grinding the cells with glass powder in the apparatus of Kalnitsky, Utter and Werkman (1945). Ground glass powder was added to the cell paste in a ratio of two grams of glass to one gram of cells. This cell-glass mixture was ground. The ground material was extracted with 0.05 M phosphate buffer, pH 7.0 and cleared of glass and cell debris by centrifugation at 10,000 r.p.m. in the high speed head of a refrigerated centrifuge for 20 minutes.

An alternative preparation was made by sonic vibration in a Raytheon 9-kc. Magnetostriction Oscillator. This was the method of preference and was used in all instances in which the extract was to be fractionated with ammonium sulfate. If the extract were prepared from acetone-treated cells, 2 g. of cells, 3 g. of glass beads (0.2 millimeters in diameter) in 25 ml. 0.05 M phosphate buffer, pH 7.0 were subjected to sonic disintegration for 20 minutes. The cell debris and glass beads were removed by centrifugation at 10,000 r.p.m. in the high speed head of a refrigerated centrifuge for 20 minutes.

Cell-free extracts were prepared from fresh cells in a similar manner; 7 g. cell paste and 5 g. glass beads were suspended in 20 ml. 0.05 M phosphate buffer, pH 7.0. The remainder of the treatment was the same as that described for

extracts of acetone-treated cells.

The cup contents were kept cold during sonic treatment by circulating ice-cold water through the apparatus.

## Analytical Procedures

### Phosphate determination.

The method of Fiske and SubbaRow (1925) was used for the determination of inorganic phosphorus and total phosphorus. The color was developed by mixing the reagents with a water solution of the sample containing 4-40 micrograms phosphorus. After standing for 3.5 minutes the color intensity was read in a Klett-Summerson photoelectric colorimeter with a 660 mμ filter. The reading was referred to a standard curve prepared under identical conditions and the amount of phosphorus determined.

Total phosphorus was determined by ashing the sample. The sample was heated in a micro-Kjeldahl flask with 0.3 ml. concentrated $H_2SO_4$ until white fumes of $SO_3$ appeared. This was followed by the addition of 2-3 drops concentrated $HNO_3$. Heating was continued until the brown fumes of $NO_2$ appeared. Finally 3 drops of $H_2O_2$ were added and heating was completed in 5 minutes. The sample was then diluted and an analysis for inorganic phosphorus was carried out.

The Lowry and Lopez (1946) method as described by Kalckar (1947d), was used to determine inorganic phosphorus in the presence of labile organic phosphoric acid esters. To avoid losses of labile organic phosphorus in the perchloric acid deproteinization, the mixtures were partially neutralized by adding sodium acetate solution as specified, but prior to centrifugation. By this modification the samples were exposed to perchloric acid not longer than 1 minute. The removal of protein may be less complete, but no interference with the phosphorus determination was observed. The color was developed in 3 minutes and the color intensity was read in a Klett-Summerson photoelectric colorimeter at 660 mμ . The phosphorus was determined by reference to a standard curve prepared under identical conditions. The range was 2-12 micrograms of phosphorus per sample.

Nitrogen determination.

Nitrogen was determined by the micro-Kjeldahl method. $CuSO_4$-$K_2SO_4$ catalyst was used. The digested samples were steam-distilled in a Parnas-Wagner still or in a Kork microstill and collected in either 4% boric acid if the titration method were to be used, or in 0.01N HCl if a colorimetric method were to be used.

Titration method. The sample in 4% boric acid was titrated with 0.01N or 0.01N HCl (depending upon the amount of nitrogen present) using a mixed indicator (0.125% methyl red and 0.083% methylene blue in 95% ethanol).

Colorimetric methods. (1) Nesslerization. Of the various methods for the preparation of Nessler's reagent, the procedure given in Pierce and Haenisch (1940, p. 412) proved to be the most satisfactory. The reagent was prepared by dissolving 13 g. KI in 25 ml. ammonia-free water. Cold, saturated $HgCl_2$ was then added with constant stirring until the precipitate that first forms no longer redissolves and the mixture was filtered through paper. A solution of alkali was prepared by dissolving 55 g. KOH is 150 ml. water; the precipitate of carbonate was allowed to settle and the supernatant was decanted. The KOH wolution was then added to the filtered solution of KI and $HgCl_2$, the volume was made up to 250 ml. with distilled water and mixed. Saturated $HgCl_2$ solution was added drop by drop until a slight permanent precipitate is formed. With this reagent the color was developed by treating the sample containing 20-150 micrograms ammonia nitrogen in the presence of gum arabic. The color intensity was determined in a Klett-Summerson photoelectric

colorimeter at 490 mμ and referred to a standard curve prepared under identical conditions. Despite the use of gum arabic to stabilize the mixtures, colloidal material often appeared rendering the determination worthless. The method was later abandoned in favor of the Russel phenol-hypochlorite method.

(2) Phenol-hypochlorite method of Russell (1944). A 1.5 ml. sample in neutral or acid solution (not stronger than 0.01-0.02N) containing 0.5-6.0 micrograms ammonia nitrogen was placed in a tube cooled by an ice bath. To this was added 1 drop manganous salt solution (0.003N $MnCl_2$ or $MnSO_4$), 1.0 ml. cold alkaline phenol reagent (25 g. crystalline phenol, dissolved with stirring in 54 ml. 5.0N NaOH made up to 100 ml. with water) and 0.5 ml. cold sodium hypochlorite solution (chlorox diluted to contain 1.30-1.40% free chlorine), and the contents were mixed by gentle rotation. The tube placed in a boiling water bath for 5 minutes, then cooled and diluted to 6.0 ml. The color intensity was measured at 625 mμ in a Beckman spectrophotometer and compared to a standard treated in the same manner.

Pentose determination.

Pentose was determined by the method of Mejbaum (1939).

To a 1.5 ml. water solution of the deproteinized sample (containing 1-15 micrograms pentose) was added 1.5 ml. of orcinol reagent (0.1% $FeCl_3$ in concentrated HCl containing 1% orcinol). The mixture was heated for 40 minutes in a boiling water bath, cooled, and the volume adjusted to 5.0 ml. with distilled water. The color intensity was read at 660 mμ in a Klett-Summerson photoelectric colorimeter. Commercial orcinol preparations often contain impurities which reduce the final color intensity of the test giving erroneous results. Orcinol must be purified by recrystallization from chloroform before use with the method.

Ribose when bound to the pyrimidine nucleosides and nucleotides shows limited response to the orcinol reagent of Mejbaum. Table 1 shows the response of several of these compounds. Even the most reactive compound, uridine, gives only about 10% of the expected color intensity. Steam distillation under a rigidly controlled and elaborately designed system has been used by Dunstan and Gillam (1949) and Barker (1950) to increase the recoveries in ribose from pyrimidine nucleosides and nucleotides. It is known that removal of the double bond between carbons 4 and 5 in the pyrimidine ring and substitution with hydrogen according to Levene and LaForge (1912) or with bromine after the method of Massart and Hoste (1947), render the bond between the pyrimidine base and ribose more labile and the compound

Table 1.

Response of pyrimidine ribose compounds to orcinol reagent.

| Substance examined | Concentration micro-moles | $E_{(660m)}$ observed |
|---|---|---|
| Ribose | 0.023 | 0.105 |
| Ribose | 0.045 | 0.220 |
| Ribose | 0.057 | 0.278 |
| Ribose | 0.086 | 0.440 |
| Cytidine | 0.248 | 0.013 |
| Cytidine | 1.013 | 0.140 |
| Uridine | 0.266 | 0.101 |
| Uridine | 0.665 | 0.643 |
| Cytidylic acid | 0.233 | 0.006 |
| Cytidylic acid | 1.032 | 0.058 |
| Uridylic acid | 0.204 | 0.041 |
| Uridylic acid | 1.018 | 0.423 |

Experimental conditions: Mejbaum orcinol test for pentose; the procedure is outlined in the text.

responds to the Mejbaum pentose test.

Various attempts were made to make the pyrimidine ribosides responsive to the Mejbaum orcinol test by catalytic hydrogenation. Raney nickel aluminum catalyst with hydrogenation was tried; however, upon addition of the acid orcinol reagent hydrogen was evolved and the color intensity anticipated was never reached. This might have been due to reduction of the ferric chloride catalyst to the ferrous state. In order to preclude the presence of hydrogen, the mixture was acidified and the hydrogen evolved from the action of the acid on the catalyst removed prior to the addition of orcinol reagent. This attempt also failed; again the color never reached the expected intensity. A palladium catalyst (Baker Colloid 46) was also used. Brown precipitates in the heated test caused this approach to be discarded. With the lack of success in the attempts to solve the problem by catalytic hydrogenation, variations in the Mejbaum test were explored. Various heating times and acid concentrations were tried. The response was more favorable. It was found that the following conditions gave the best results. The orcinol reagent used was the same as that described above. To the sample (0.1 micromole or less) in 1.0 ml. water, 2.5 ml. concentrated HCl and 1.5 ml. orcinol reagent were added. This was heated in a boiling water bath for 240 minutes. After cooling, the volume was adjusted to

5.0 ml. with distilled water. The development of the color is shown in Figure 1. Some of the acid is lost during the heating period. The acidity changes from about 9N to 8N. The absorption maximum is located at 670 mμ as it is in the Mejbaum pentose determination. As in the Mejbaum test, glucose and glucosidic compounds interfere, causing a brownish-yellow color which masks the green color desired. The color obtained is stable. For routine tests the Klett-Summerson photoelectric colorimeter with a 660 mμ filter was used. A Beckman spectrophotometer, model D, was used for the spectrophotometry. Some discussion of the possibilities and limitations of the method may be desirable. As is shown in Figure 1 the response is not uniform. The modified test is only semiquantitative for mixtures of pyrimidine ribose derivatives. It can be used for quantitative determinations if only one compound is present in the sample and the same substance is used as a reference standard. The procedure is recommended solely for estimation of pyrimidine bound ribose. It does not supersede the Mejbaum method for pentose determinations under ordinary circumstances. Choice of the correct orcinol method makes possible the following measurements:

(a) Quantitative determination of pyrimidine ribose nucleosides or nucleotides with the same material as reference (modified procedure).

(b) Check for completeness of metabolism of pyrimidine-bound ribose in enzyme systems (modified procedure).

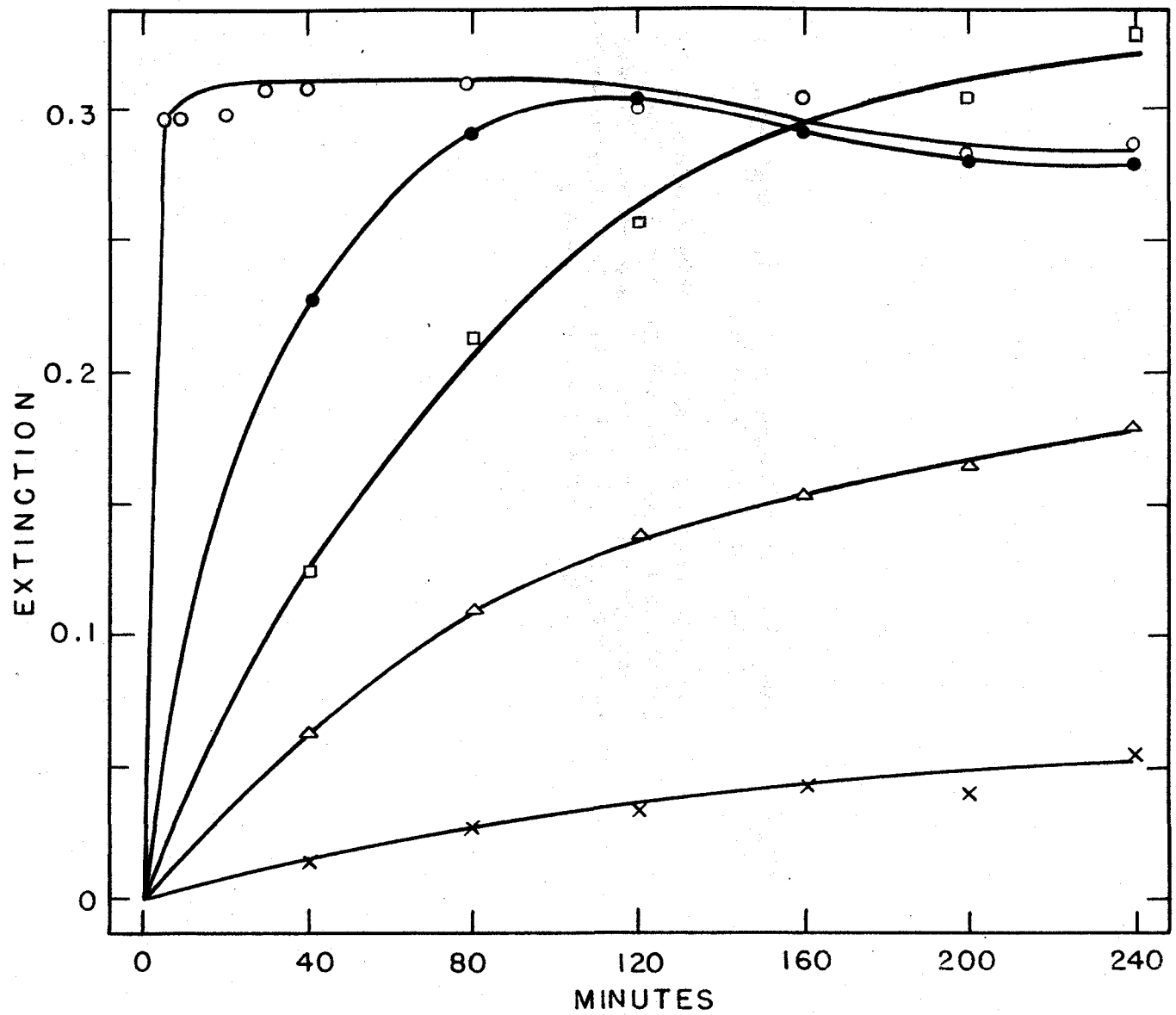
(c) Distinction between pyrimidine-bound and other ribose derivatives (purine-bound ribose, ribose phosphoric acid esters, free ribose) by a combination of the two procedures.

Protein determination

The biuret method of Weichselbaum (1946) was used to determine protein quantitatively in cell-free extracts. The color was developed by adding the reagent to a saline solution (0.85% NaCl) of the extract containing 0.5-2.0 mg. protein nitrogen, and incubating at 30°C. for 30 minutes. The intensity of the color was read in a Klett-Summerson photoelectric colorimeter at 540 mμ. A standard curve was prepared using a cell-free extract of E. coli as a source of protein. The protein of this extract was determined by a micro-Kjeldahl digestion, steam distillation, and titration of the ammonia nitrogen with standard acid. Since proteins vary with the source, the biuret test must be standardized with a protein similar in nature to the protein material which will be tested later.

Fig. 1. Response of pyrimidine ribose compounds to modified orcinol reagent.

Experimental conditions: Concentration of substances, 0.1 micromole per ml. The procedure is outlined in the text. Curve: (o) ribose; (•) uridine; (□) uridylic acid; (△) cytidine; (x) cytidylic acid.


EXTINCTION
0.3
0.2
0.1
0
0
40
80
120
160
200
240
MINUTES

Hypoiodite titration.

The metabolism of purine ribosides and pyrimidine desoxyribosides was followed by means of a hypoiodite titration. (Willstatter and Schudel, 1919; MacLeod and Robison, 1929; Grynberg, 1932; Dmochowski, Zajdenman and Rabanowska, 1935) An excess of standard iodine was added to an aqueous solution of the substances to be determined. Alkali was added dropwise in the approximate ratio of 3 equivalents of NaOH to 2 equivalents of iodine. The container was then stoppered and allowed to stand in the dark for 1 hour. The solution was then acidified and the excess iodine titrated with standard sodium thiosulfate. Klein (1935) has shown that the following compounds take up the equivalents of iodine indicated: ribose, 2 equivalents; desoxyribose, 2 equivalents; adenine, 0 equivalents; guanine, 4 equivalents. Bagatell (1951) found that hypoxanthine, uracil, cytosine, and thymine take up no iodine.

Spectrophotometry

The Beckman spectrophotometer, model D, was used to carry out all spectrophotometric measurements in the visible light range; model DU was used to carry out ultra-violet spectrophotometric measurements. Enzymatic changes which permitted the technique of differential spectrophotometry

were followed by the methods outlined by Kalckar (1945; 1947a,b,c).

Uracil was determined spectrophotometrically according to Wang, Sable and Lampen (1950). The deproteinized, neutralized sample was made 0.1N with NaOH and read at 300 mμ . Later this procedure was replaced by the method of Carter (1951). The adsorption at 290 mμ in 0.01N NaOH was used. The results seemed to be more reproducible when the latter conditions were used. Both methods are based on earlier work reported by Hotchkiss (1948) and by Ploeser and Loring (1949) which show that uracil adsorbs much more strongly than does uridine at the wave length and pH indicated.

## Preparation of Compounds

Cytidylic acid, uridylic acid, and uridine were commercial products. Their identity was verified by spectrophotometric methods. Cytidine was prepared by hydrolysis of commercial ribose nucleic acid by the method of Brederek et al. (1941). The product was recrystallized as the sulfate (N 14.7%, calculated 14.35%; $E_m$(270 mμ ) = 8.7 x $10^3$). Thymine desoxyriboside was generously furnished by Dr. E. E. Snell, University of Wisconsin. Adenosine and guanosine were prepared from commercial ribose nucleic acid by the

method of Bredereck et al. (1941). The compounds were checked for purity spectrophotometrically and by pentose determination. Inosine was prepared by enzymatic deamination of adenosine with calf intestinal deaminase purified according to the method of Brady (1942). Uracil was a commercial product. It was recrystallized from water prior to use. The purity of the recrystallized compound was checked spectrophotometrically. Cytosine was prepared by formic acid hydrolysis of cytidine according to Vischer and Chargaff (1948) and isolated via the phosphotungstate (N 33.7%, calculated 32.3%; $E_m$(262 mμ ) = 6.6 x $10^3$). Orotic acid was synthesized according to the method outlined by Muller (1897) and by Mitchell and Nye (1947) (N 15.9%, calculated 16.1%; $E_m$ (277 mμ ) = 7.3 x $10^3$). Thymine was a commercial product. Its identity was verified by spectrophotometric measurements.

Isolation of ribose-1-phosphate.

Ribose-1-phosphate was isolated by an adaptation of the method of Friedkin (1950). An excess of uridine was incubated with the purified enzyme for 3 hours at 37°C. At the end of the incubation period 1.7 times as many millimoles[1] of magnesia reagent (0.5M $MgCl_2$ - 5.0N $NH_4Cl$) as

---

[1]Calculation based on the concentration of magnesium.

millimoles of inorganic phosphorus present in the reaction mixture were added. Sufficient concentrated $NH_4OH$ was then added to make the entire solution approximately 0.42N with respect to $NH_4OH$. This mixture was then refrigerated for 3 hours. The precipitate of $MgNH_4PO_4$ was removed by filtration through a pad of cotton and paper pulp. To the clear filtrate 1.3 times as many millimoles of ammoniacal barium acetate mixture (2.0 g $Ba(C_2H_2O_2)_2 \cdot H_2O$, 10 ml. water, 0.6 ml. 15N $NH_4OH$), on the basis of barium acetate, as millimoles of labile phosphorus anticipated were added and the mixture allowed to stand overnight in the refrigerator. Any precipitate which formed was spun down and discarded. To the clear supernatant 4 volumes of cold 95 per cent ethanol containing 0.4 ml. 15N $NH_4OH$ per 46 ml. supernatant was added. After standing for 10 hours in the refrigerator, the barium salt which formed was removed by centrifugation. The precipitate was washed once each with 95 per cent alcohol, absolute alcohol, and ether and dried in a vacuum desiccator. The ribose-1-phosphate may be purified by dissolving it in water, removing any insoluble substance by centrifugation and reprecipitating with alcohol as described above.

# EXPERIMENTAL RESULTS

## Studies of the Metabolism of Pyrimidine Ribosides

Many of the concepts regarding pyrimidine riboside metabolism have been deduced by analogy to the metabolism of purine ribosides. The metabolism of purine ribosides by E. coli has been demonstrated by Lutwak-Mann (1936) and by Stephenson and Trim (1938). Purine ribosides were cleaved by the organisms; free purine base was liberated, and the pentose formed was dissimilated and disappeared rapidly from incubation mixtures. The metabolism of purine and pyrimidine desoxyribosides has also been studied by Manson and Lampen (1947) who demonstrated that it proceeded in a fashion similar to that of the purine ribosides.

### Metabolism of ribose.

It was found that washed, whole cells of E. coli and A. aerogenes are capable of metabolizing pyrimidine nucleosides and nucleotides. The ribose formed was immediately dissimilated; none was accumulated in the reaction mixture. Deamination of the molecules containing aminonitrogen groups took place simultaneously; ammonia was detected in the reaction mixture. This is illustrated in Table 2. The amounts of ribose metabolized exceed the ribose content of

Table 2.

Metabolism of Pyrimidine Ribosides by E. coli and A. aerogenes.

| Substrate | % Ribose metabolized | | % Deamination | |
|---|---|---|---|---|
| | E. coli | A. aerogenes | E. coli | A. aerogenes |
| Cytidine | 95 | 97 | 72 | 76 |
| Uridine | 100 | 92 | -- | -- |
| Cytidylic acid | 89 | 85 | 79 | 83 |
| Uridylic acid | 100 | -- | -- | -- |

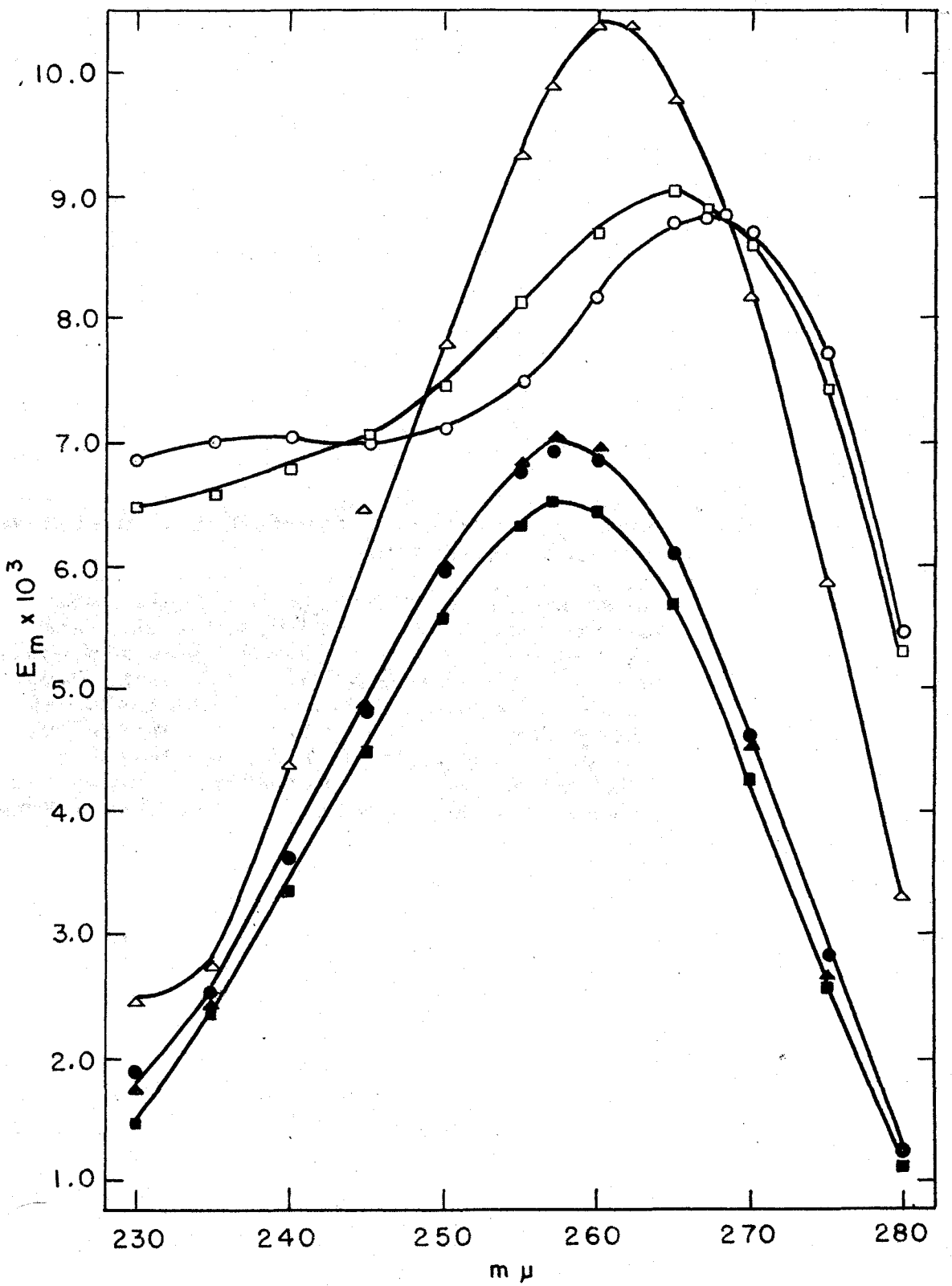
Experimental conditions: cytidine, 2.9 micromoles per ml.; uridine, 3.4 micromoles per ml.; cytidylic acid, 2.6 micromoles per ml., uridylic acid, 2.6 micromoles per ml.; phosphate buffer, 0.03M, pH 7.0; 17 mg. fresh cells per ml.; t = 37°C.; time of reaction, 4 hours.

the cells many fold on a molar basis. In all instances the endogenous ribose metabolism remained below 0.3 micromoles per ml. The endogenous ribonucleic acid did not interfere with the pentose tests since it did not diffuse out of the cells during the incubation period. The disappearance of ribose from the incubation mixture was not due to retention of it in the bacterial cell. This was ascertained by orcinol tests of the samples without removal of the bacterial cells. The amount of ammonia formed was less than expected from ribose metabolism. It would appear that part of it was retained by the cells. The values presented in Table 2 were corrected for endogenous ammonia formation (0.5 micromoles per ml. for E. coli and 1.6 micromoles per ml. for A. aerogenes). This experiment did not permit a decision as to whether deamination occurred at the nucleoside stage or after liberation of cytosine. It was later observed (experiments with pyrimidine nucleoside phosphorylase) that cytidine was not cleaved; thus it would seem that deamination of the nucleoside occurs.

The pyrimidine nucleus was not metabolized under the experimental conditions employed, except for the deamination of the cytosine derivatives. The accumulation of uracil was demonstrated spectrophotometrically (see Figure 2). The absorption maximum shifted during incubation from 266 mμ (cytidine) and 268 mμ (cytidylic acid) to 258 mμ , and

Fig. 2. Spectrophotometric Observation of Pyrimidine Riboside Metabolism.

Experimental conditions: cytidine, 2.85 micromoles per ml.; cytidylic acid, 2.85 micromoles per ml.; uridine, 2.85 micromoles per ml.; phosphate buffer, 0.1 M, pH 7.0; cells, 17 mg. per ml.; t 37°C.; time of incubation, 4 hrs.; Curve: (□) cytidine, 0 hrs.; (○) cytidylic acid, 0 hrs.; (△) uridine, 0 hrs.; (■) cytidine, 4 hrs.; (●) cytidylic acid, 4 hrs.; (▲) uridine, 4 hrs.


10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
$E_m \times 10^3$
230
240
250
260
270
280
m μ

that of uridine from 261 mμ to 258 mμ which indicates formation of uracil in all cases. For a comparison, the spectrophotometry of the pure substances is shown in Figure 3. It will be observed that a discrepancy exists between the absorption intensity of pure uracil and that obtained after incubation. Two interpretations can be offered: (1) a small part of the uracil is retained by the cells; and (2) a less plausible explanation, some of the uracil is metabolized.

Experiments with the Warburg respirometer (see Table 3) showed that the ribose was oxidized to carbon dioxide and water. The oxidation was not complete at the end of 210 min., when the reaction was interupted. In experiments on the metabolism of cytidine in which the modified pentose test was used to follow the reaction, no response was obtained after incubation for 30 min. (98% of the substrate had disappeared by this time). This indicates the temporary accumulation of orcinol-negative intermediates.

The pH optimum for ribose metabolism was studied and found to be 7.0. There was a gradual decline of activity at higher pH and a rapid decrease at pH values below 6.5. Actively metabolizing whole cells make the determination of a pH optimum difficult. Much acid is produced by the organisms and unless excessively high concentrations of buffer are used, the pH gradually lowers as the incubation pro-

Fig. 3. Spectrophotometry of Pure Pyrimidine Compounds.

Experimental conditions: cytidine, 23.5 micromoles per absorption cell; cytidylic acid, 21.5 micromoles per cell; uridine, 20.3 micromoles per cell; uracil, 11.5 micromoles per cell; phosphate buffer, 0.02M, pH 7.0; total volume, 3.0 ml. Curve: (△) cytidine; (○) cytidylic acid; (□) uridine; (●) uracil.

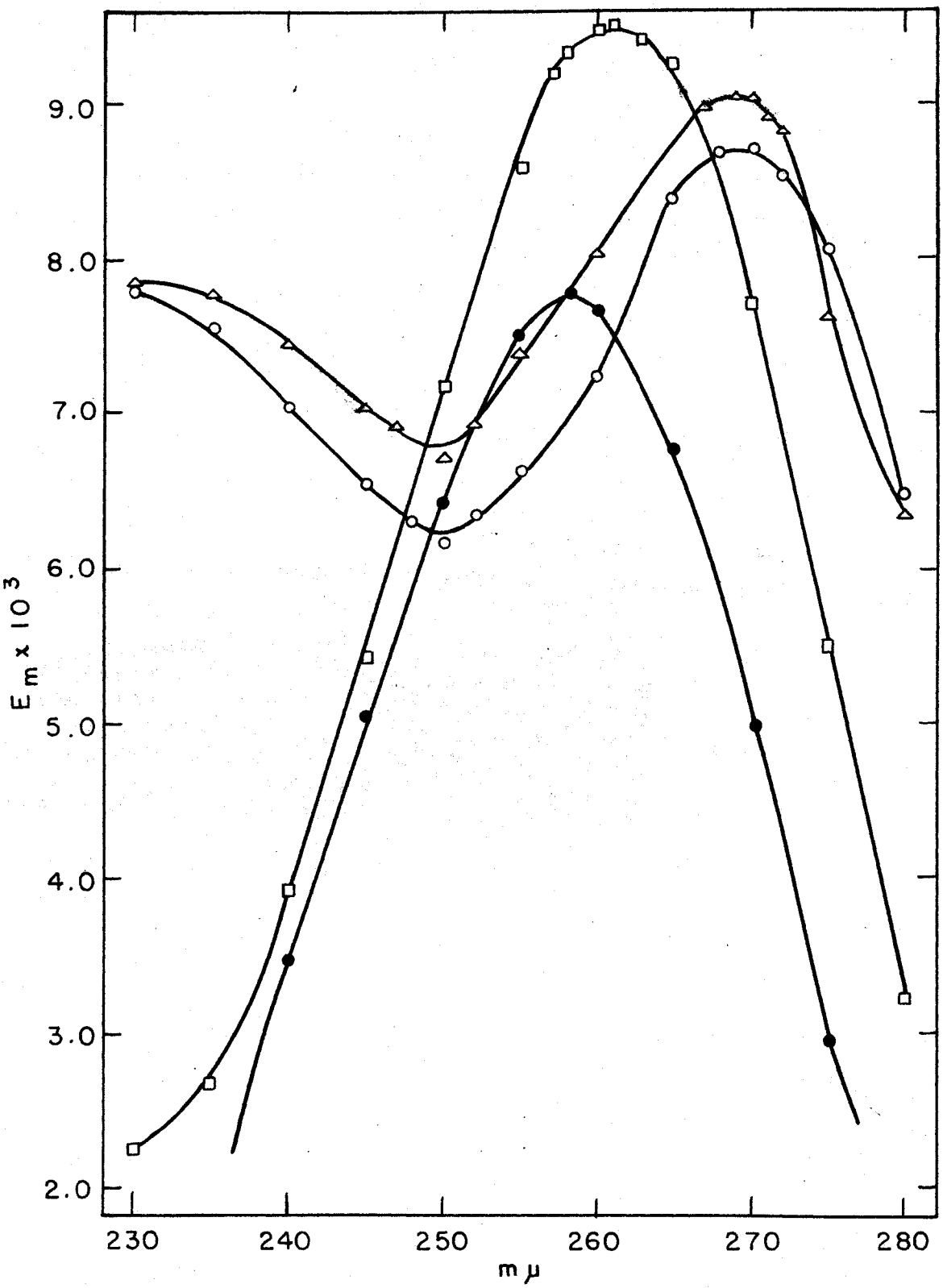

9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
$E_m \times 10^3$
230
240
250
260
270
280
mμ

Table 3

Metabolism of Uridine and Cytidine by *E. coli*.

| Substrate | Microliters Oxygen Uptake | | | | |
|---|---|---|---|---|---|
| | 30' | 60' | 120' | 180' | 210' |
| Endogenous | 39 | 83 | 172 | 242 | 274 |
| Uridine | 79 | 126 | 349 | 465 | 520 |
| Cytidine | 89 | 137 | 338 | 430 | 472 |

Experimental conditions: Uridine, 2.0 micromoles per ml.; cytidine, 1.9 micromoles per ml.; phosphate buffer, 0.03M, pH 7.0; fresh cells, 25 mg. per ml.; manometric technique, 0.3ml. 10%. NaOH in center well; total volume, 2.0 ml.; t = 30.4°C.

gresses. This was not completely overcome in this experiment.

Role of phosphorus.

The role of phosphate ion in the metabolism of purine ribosides has been established by Klein (1935a) and by Kalckar (1945a,b; 1947c,d). At the time the following work was performed an analogous function of phosphate for pyrimidine ribose metabolism had not yet been demonstrated. Since then several workers have shown that the enzymes of pyrimidine riboside and desoxyriboside metabolism are also phosphorylytic (Manson and Lampen, 1951b).

Attempts were made to determine the role of phosphorus in the metabolism of pyrimidine nucleosides by whole cells. E. coli must be grown in a medium highly buffered with phosphate in order that maximum nucleolytic activity be obtained. Sufficient phosphate was carried along with the thoroughly washed cells to render the experiments inconclusive. M. lysodeikticus was used in an effort to resolve the problem, since the medium on which it is cultured is low in phosphate. These organisms failed to metabolize cytidine, however. Permeability might have been a contributing factor.

Experiments with acetone-treated cells of E. coli

offered better prospects. Ribose metabolism was arrested in an early stage when acetone-treated cells were used. In contrast to experiments with whole cells in which the ribose was rapidly metabolized; ribose accumulated in mixtures in which a nucleoside was incubated with a suspension of acetone-treated E. coli cells (see Figure 4). This was very convenient, because the ordinary pentose method of Mejbaum (1939) could be used to follow the reaction.

The data in Table 4 suggest strongly that the rate of splitting of cytidine and uridine depends on the phosphate concentration. As with the experiments with whole cells, the influence shown by phosphate is not conclusive since considerable amounts of the nucleosides were metabolized in the absence of added phosphate ion. Here again the possibity that phosphate was carried along with the cells through acetone-treatment exists.

The accumulation of ribose-1-phosphate could be measured with the Lowry and Lopez method (1946) for the determination of labile phosphoric acid esters. This is demonstrated in Figure 5. The identity of the labile phosphoric acid ester with ribose-1-phosphate is suggested by its mode of formation, its lability toward acid, and its response in the pentose test. No significant esterification of phosphate could be found with the Fiske and SubbaRow method (1925).

Fig. 4. Metabolism of Cytidine by Acetone-Treated Cells of *E. coli*.

Experimental conditions: cytidine, 2.72 mg. per ml.; phosphate buffer, 0.03M, pH 7.0; acetone-treated cells, 6.6 mg. per ml.; t = 37°C.

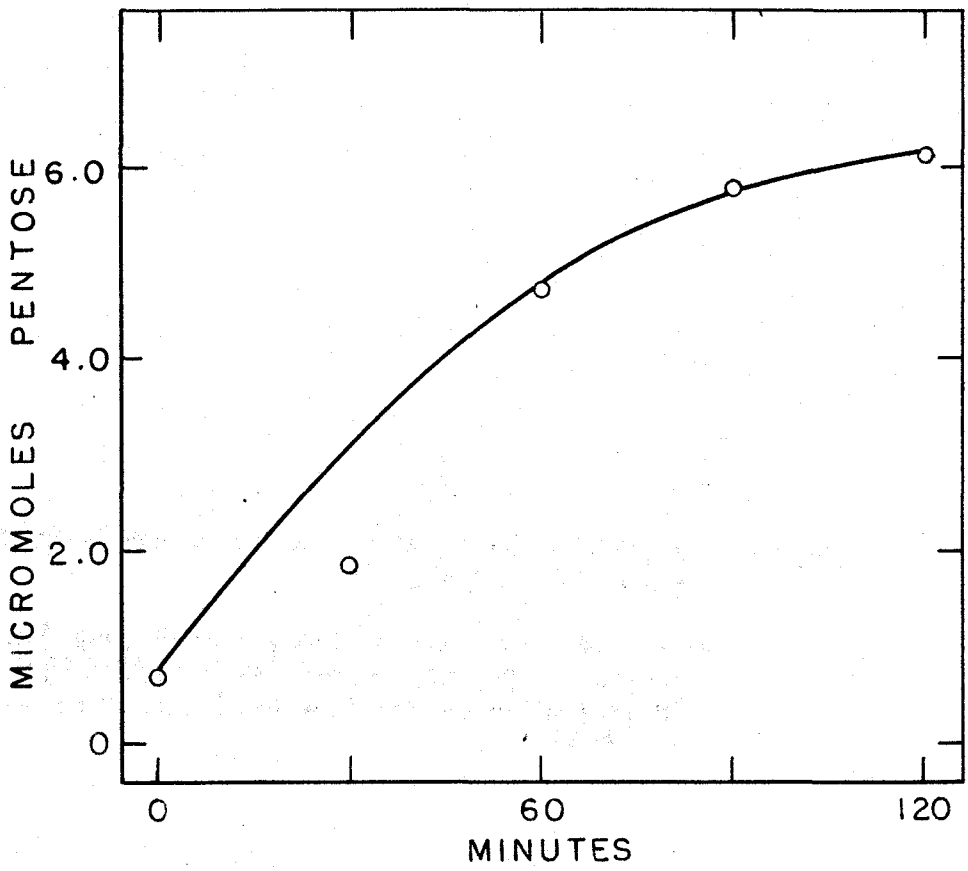

MICROMOLES PENTOSE
6.0
4.0
2.0
0
0
60
120
MINUTES

Table 4.

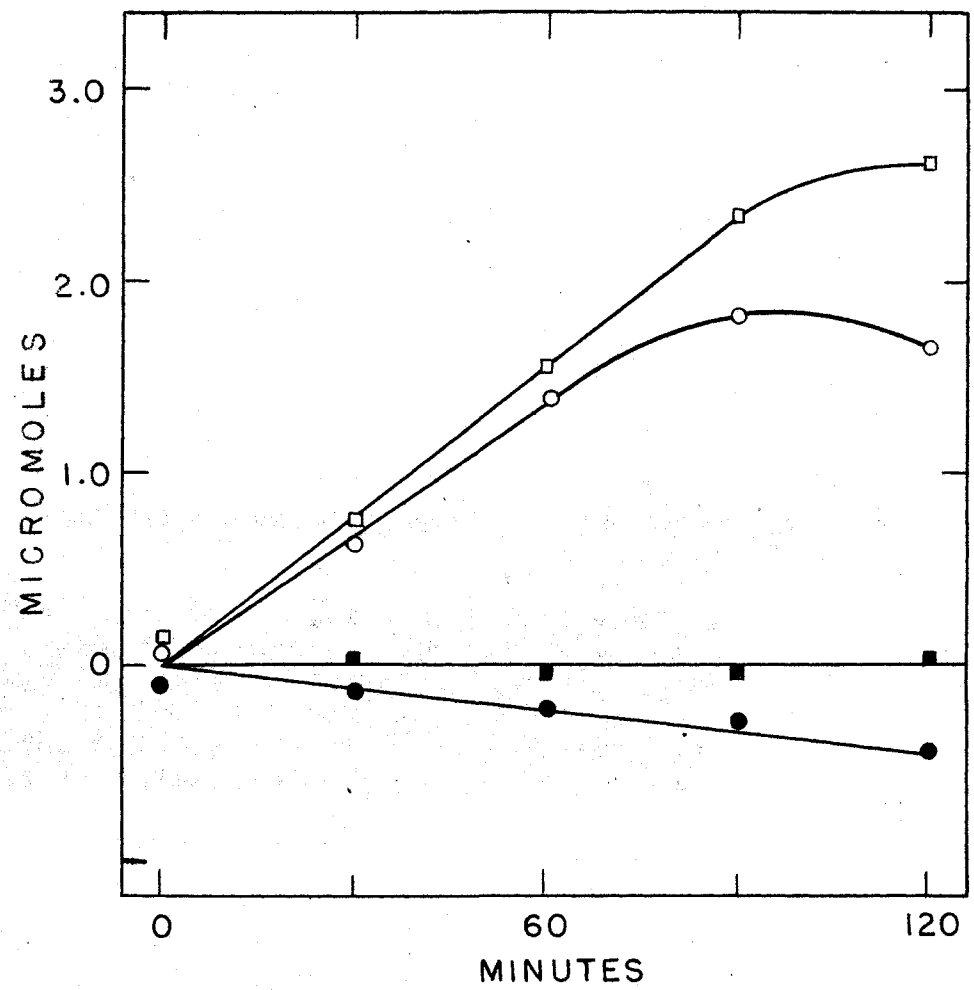
Role of Phosphate in the Enzymatic Splitting of Pyrimidine Ribosides

| Substrate | Phosphate concentration | Micromoles ribose | |
|---|---|---|---|
| | | 0' | 90' |
| Cytidine | 0.066 M | 0.13 | 3.41 |
| Cytidine | 0.01 M | 0.24 | 3.09 |
| Cytidine | $H_2O$ | 0.16 | 1.67 |
| Uridine | 0.066 M | 1.09 | 3.37 |
| Uridine | 0.01 M | 0.88 | 2.91 |
| Uridine | $H_2O$ | 0.68 | 1.75 |

Experimental conditions: cytidine, 4.5 micromoles per ml.; uridine, 4.5 micromoles per ml.; phosphate buffer, pH 7.0; acetone-treated cells, 6.6 mg. per ml.; t = 37°C.

Fig. 5. Formation of Ribose-1-Phosphate from Cytidine.

Experimental conditions: cytidine, 4.5 micromoles per ml.; phosphate buffer, 0.01M, pH 7.0; acetone-treated cells, 6.6 mg. per ml.; t 37°C. Curve: (▫) pentose liberated; (▪) pentose control; (○) ribose-1-phosphate formed; (●) ribose-1-phosphate control.


3.0
2.0
1.0
0
MICROMOLES
0
60
120
MINUTES

Specificity of pyrimidine nucleosidase.

The foregoing experiments showed that in acetone-treated cells of E. coli the breakdown of pyrimidine ribosides was interrupted after the nucleosidase reaction. The following equilibrium is established:

Pyrimidine riboside + phosphate $\rightleftharpoons$
pyrimidine base + ribose-1-phosphate

Attempts were made to test which pyrimidine bases might be components of this equilibrium. Only pyrimidine compounds capable of reacting with ribose-1-phosphate might be expected to influence the reaction rate by shifting the equilibrium. To eliminate permeability effects a cell-free nucleosidase preparation was used. An extract obtained by subjecting acetone-treated cells to sonic disintegration, metabolized both uridine and cytidine (see Figure 6). This extract was treated with saturated ammonium sulfate solution. The fraction precipitated from 0-0.75 saturation was removed by centrifugation (10,000 r.p.m. for 20 min. in the high speed head of a refrigerated centrifuge), dissolved in a minimum amount of water, and dialyzed overnight. The results obtained by incubating uridine with and without an excess of pyrimidine bases is shown in Table 5. It may be seen that the excess of uracil retards the splitting of

Fig. 6. Metabolism of Cytidine and Uridine by a Cell-Free Extract from Acetone-Treated Cells of *E. coli*.

Experimental conditions: cytidine, 4.22 micromoles per ml.; uridine, 4.49 micromoles per ml.; phosphate buffer, 0.02M, pH 7.0. Curve: (o) cytidine; (□) uridine.

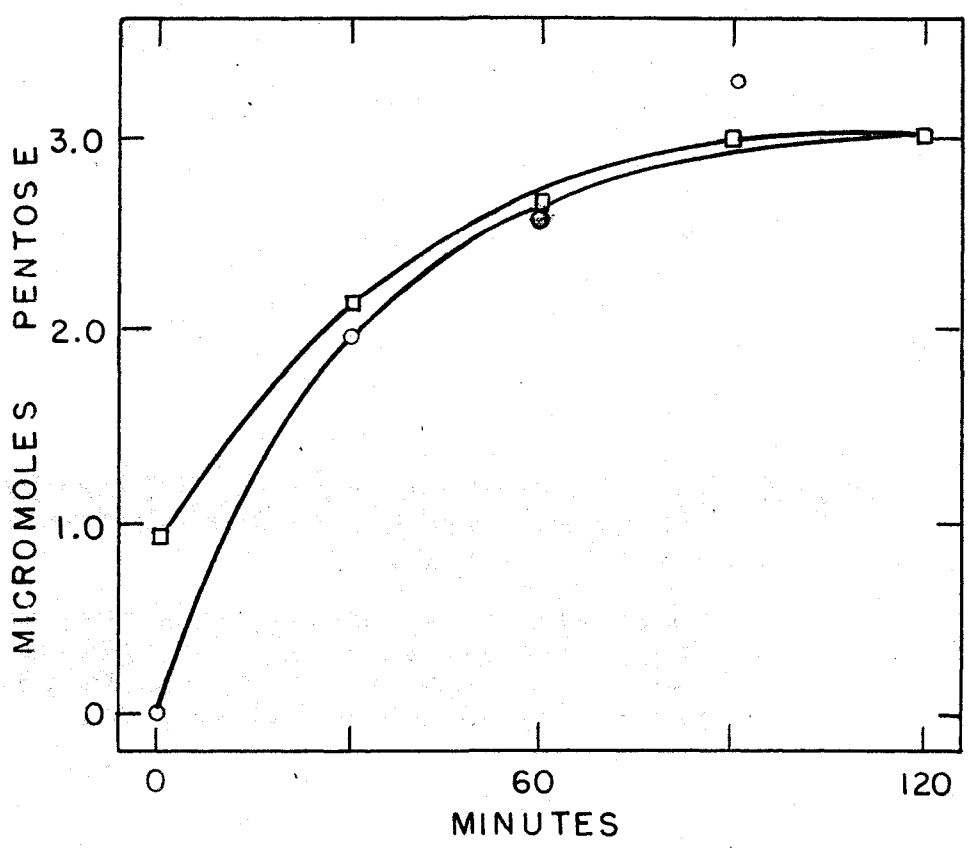

MICROMOLES PENTOSE
3.0
2.0
1.0
0
0
60
120
MINUTES

Table 5.

Influence of Pyrimidine Compounds on Pyrimidine Nucleosidase Reaction

| Substrate | Micromoles uridine split | |
|---|---|---|
| | 15' | 45' |
| Uridine | 1.27 | 1.47 |
| Uridine + uracil | 0.62 | 0.97 |
| Uridine + cytosine | 1.37 | 1.56 |
| Uridine + orotic acid | 1.23 | 1.42 |

Experimental conditions: uridine, 4.5 micromoles per ml.; uracil, 6.7 micromoles per ml.; cytosine 6.0 micromoles per ml.; orotic acid, 6.7 micromoles per ml.; phosphate buffer, 0.01 M, pH 7.0; enzyme preparation, 0.016 mg. protein-nitrogen per ml., t = 37°C.

uridine while cytosine and orotic acid are without effect. It thus appears that, in E. coli, amination and deamination of the pyrimidine nucleus take place only if the latter is combined with ribose.

The function of orotic acid in the system was further investigated. The possibility existed that an orotic acid riboside was formed under the conditions of the experiment described above; and, that this riboside (due to the presence of the carboxyl group in position four) would respond to the Mejbaum pentose test (1939). This has now been shown to be the case (Michelson, et al., 1951). Under such circumstances the results of this experiment would give no indication of the role of orotic acid. To pursue this point, ribose-1-phosphate was prepared by incubation of uridine with nucleosidase. The esterification was traced by the method of Lowry and Lopez (1946). The phosphate uptake had come to an end after 40 min. and the concentration of ester was 0.82 micromoles per ml. At this time orotic acid to a concentration of 8.0 micromoles per ml. was added. Twenty minutes later the concentration of ribose-1-phosphate was 0.81 micromoles per ml. Orotic acid, therefore, had not changed the equilibrium. It was also possible that orotic acid was first decarboxylated to uracil which was subsequently converted to uridine. To test this alternative, orotic acid was incubated in Warburg cups with fresh cells,

lyophilized cells, acetone-treated cells and a cell-free juice of E. coli. In no instance was decarboxylation observed.

## Pyrimidine Nucleoside Phosphorylase

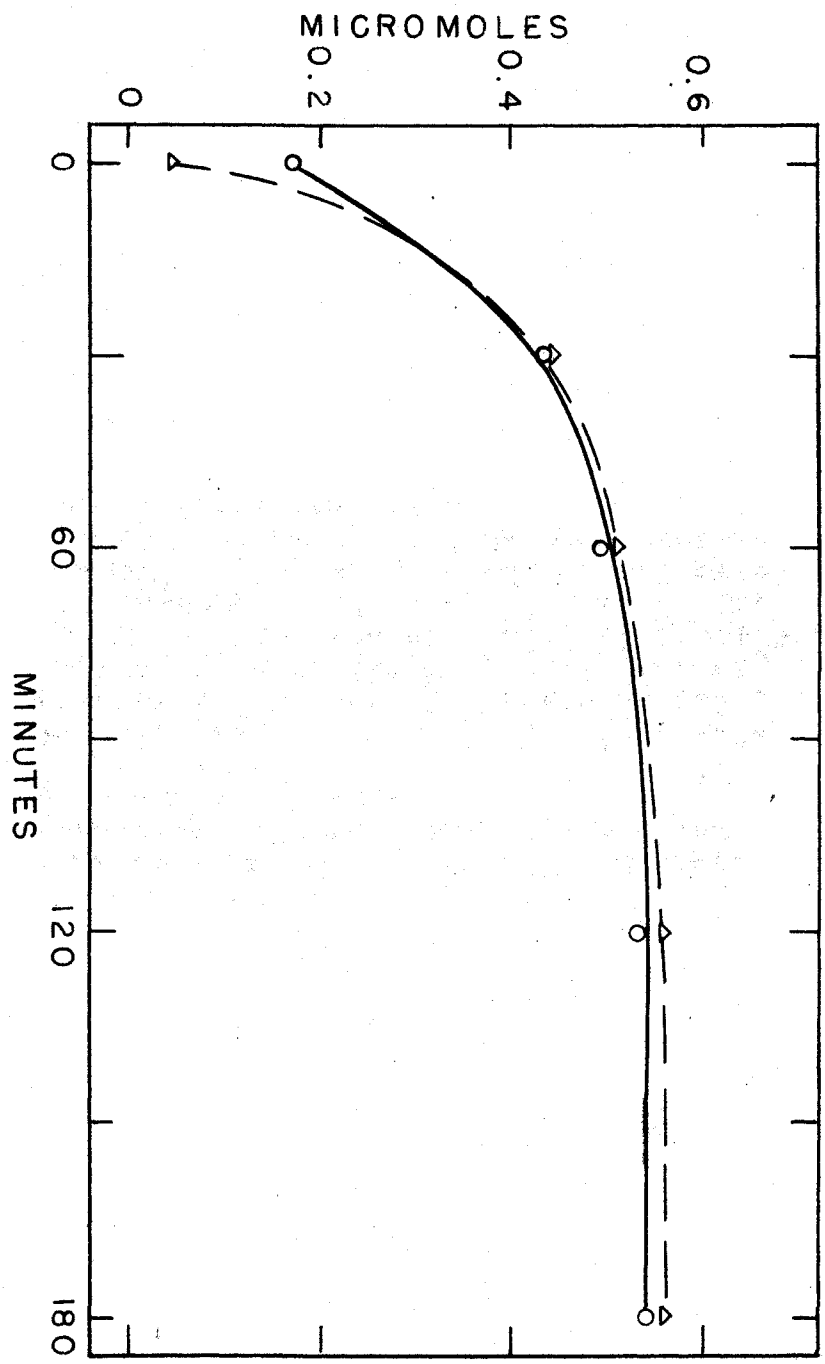
### Purification of the enzyme.

Purification of the enzyme was accomplished by fractional precipitation with saturated ammonium sulfate and by adsorption on and elution from alumina C-gamma.

Two methods were used for the determination of the nucleosidase activity of the various fractions and preparations. In early experiments the pentose test of Mejbaum (1939) was used; the amount of pentose accumulated was taken as an indication of the amount of nucleoside split. Later the spectrophotometric test of Carter (1951) was employed; the amount of uracil liberated was taken as indicative of the amount of nucleoside cleaved. Figure 7 shows the comparison of the two methods used. For reasons not clearly understood, uridine at zero time gives values somewhat higher than the ten per cent splitting usually shown due to the hydrolysis of the nucleoside under the acid conditions of the pentose method. The values were corrected for the response of the non-metabolized uridine.

Fig. 7. Uridine Splitting by Purified Enzyme. Comparison of Spectrophotometric and Colorimetric Methods.

Experimental conditions: uridine, 1.01 micromoles per ml.; phosphate buffer, 0.005M, pH 7.0; glycyl-glycine buffer, 0.03M, pH 7.0; enzyme, 0.03 mg. protein-nitrogen per ml.; total volume, 21 ml.; t = 37°C. Curve: (△) spectrophotometric method for uracil; (○) Mejbaum pentose method (colorimetric).


MICROMOLES
0
0.2
0.4
0.6
MINUTES
0
60
120
180

In order that the various fractions and preparations might be compared with respect to nucleosidase activity, a nucleosidase activity quotient, $Q_n$, was established. $Q_n$ was defined as the micromoles of uridine split per milligram protein-nitrogen per hour, and was expressed in activity units.

$$Q_n = \frac{\text{micromoles of uridine split}}{\text{(mg. protein-N) (hours)}} = \text{activity units.}$$

Uridine was always used as the substrate for the determination of nucleosidase activity. An incubation period of twenty minutes was customarily employed, since it was observed that at that time the activity was still in the straight line portion of the curve. The enzyme fractions and preparations were diluted adequately, and thus the reaction rate retarded sufficiently, so that the enzyme was always saturated with respect to substrate. Figure 7 is a time curve for the enzyme. It points out that at the end of 20 minutes the rate of splitting is still in the straight line portion.

The procedure for purification was first developed for extracts prepared from acetone-treated cells. Acetone-treated cells were used since experiments had shown that the phosphoribomutase activity of such cells was retarded and that an enzyme system beyond phosphoribomutase, criti-

cal for the dissimilation of ribose was impaired; ribose accumulated because it was not metabolized. Since the ribose was present in a form responsive to the orcinol pentose test, this was used to follow the activity of the various purification steps. It was also felt that treatment with acetone might be an essential step necessary to destroy certain enzyme systems which could potentially interfere and reduce the specificity of the enzyme preparation.

Later an extract prepared from fresh cells was fractionated, and each of the fractions checked for nucleosidase activity. The purification scheme for fresh cell extracts paralleled that of extracts of acetone-treated cells; the fractions containing maximum activity were the same in both cases. Since the major portion of the nucleosidase was destroyed by acetone-treatment, fresh cells were used to prepare extracts for fractionation. The purification procedure outlined here is for the preparation of enzyme from extracts of fresh cells.

The solution of saturated ammonium sulfate used in the following purification schedule was allowed to come to equilibrium at 0-4°C, and neutralized. All calculations and additions were based on the solubility of ammonium sulfate at that temperature. The buffered, saturated ammonium sulfate solution in the second fractionation was that used by Friedkin (1950). Neutralized saturated ammonium sulfate

containing 0.11% cysteine-hydrochloride and 1% sodium-$\beta$-glycerophosphate was employed.

A cell-free extract prepared by sonic disintegration of fresh cells of E. coli (activity, 1.4 units per ml.) was brought to 0.55 saturation with neutralized, saturated ammonium sulfate solution, added gradually with gentle stirring. The precipitate (fraction 0-0.55 saturation) was removed by centrifugation in a refrigerated angle centrifuge at 5000 r.p.m. for 25 min. and discarded. The ammonium sulfate concentration of the supernatant was raised to 0.75 saturation by gradual addition of neutralized, saturated ammonium sulfate with gentle stirring. The precipitate (fraction 0.55-0.75 saturation) was removed by centrifugation (5000 r.p.m. for 25 min.) and dissolved in a minimum amount of 0.05M phosphate buffer, pH 7.0. This fraction was then dialyzed against running, cold distilled water for 4 hours. The enzyme preparation was gently agitated by shaking or gently stirred during dialysis. This was done to effect more rapid removal of the ammonium sulfate since it was observed that the enzyme was sensitive to prolonged dialysis. The activity of the dialyzed preparation (first fractionation, 0.55-0.75 saturation) was 201 units.

The dialyzed supernatant was refractionated with buffered, saturated ammonium sulfate. The precipitate of the fraction 0-0.55 saturation was removed by centrifugation

in the high speed head of a refrigerated centrifuge at 10,000 r.p.m. for 20 min. and discarded. The saturation of the supernatant was raised to 0.75. The protein precipitate (second fractionation, fraction 0.55-0.75 saturation) was removed by centrifugation (10,000 r.p.m. for 20 min. in the high speed head of the refrigerated centrifuge) and dissolved in a minimum amount of 0.05M phosphate buffer, pH 7.0. This was then dialyzed according to the procedure described above. Activity of the fraction 0.55-0.75 saturation (second fractionation) was 352 units.

The pH of the dialyzate was then reduced to 5.0-5.5 and the enzyme was adsorbed onto alumina C-gamma (Willstätter and Kraut, 1923; prepared according to Bertho and Grassman, 1938, p. 36). The adsorption was carried on for 20 min. with gentle stirring, after which the precipitate was removed by centrifugation at 10,000 r.p.m. for 5 min. The precipitate was washed once with a small volume of water and compacted by centrifugation (10,000 r.p.m. for 5 min.). The enzyme was eluted from the alumina C-gamma by gently stirring the precipitate with a convenient volume of cold, 0.2M phosphate buffer, pH 7.0 for 20 min. The precipitate was sedimented by centrifugation (10,000 r.p.m. for 5 min.). The activity of the purified enzyme was 801 units.

Phosphate ion from the elution buffer was often undesirable in the experiments contemplated. In such instances

it was removed by dialysis according to the procedure described above.

The enzyme preparation was stored in the frozen state. If the enzyme were preserved in phosphate buffer it retained approximately 85% of its original activity after storage for 7 days. However, solutions of the enzyme from which the phosphate buffer was removed by dialysis were sensitive to storage and became completely inactive after a period of 3 days.

Properties of pyrimidine nucleoside phosphorylase.

pH optimum. The pH optimum for the enzyme was established at 7.2 as indicated in Table 7. The activity of the enzyme decreases markedly at pH values below 6.5 and above 7.5. All incubations were carried out at pH 7.2. It was found that when phosphate buffer, pH 7.0, was utilized in final concentrations between 0.03M and 0.06M, the pH at the end of the incubation period was approximately 7.2. This slightly alkaline pH value corresponds to those reported previously for other pyrimidine nucleosidases. Since the activity of the enzyme varies from preparation to preparation, the data presented in Table 7 are expressed as the percent of maximum splitting. In this manner the values from two different determinations could be compared.

Table 6.

Purification of pyrimidine nucleosidase from acetone-treated and fresh cells of *Escherichia coli*.

| | Purification procedure | Acetone-treated * | Fresh * |
|---|---|---|---|
| Extract | | - | 1.4 |
| Fraction I | Neutral saturated $(NH_4)_2SO_4$ to 55% saturation | 0.7 | 8.7 |
| Fraction II | Neutral saturated $(NH_4)_2SO_4$ 55-75% saturation | 1.3 | 201 |
| | II dialyzed 4 hours against distilled water at 0-3°C. then refractionated with neutral saturated $(NH_4)_2SO_4$ containing 1.0% sodium-glycerophosphate and 0.22% cysteine-HCl. | | |
| Fraction IIa | To 55% saturation | 3.5 | 29.5 |
| Fraction IIb | 55-75% saturation | 4.3 | 352 |
| | IIb dialyzed 4 hours against distilled water at 0-3°C. then treated with alumina C-gamma (1.0 ml. alumina C-gamma to 6.0 ml. IIb for 20 min. | | |
| Fraction A C-gamma supernatant | Alumina C-gamma removed by centrifugation | 1.6 | 245 |
| Fraction A C-gamma wash | Alumina C-gamma precipitate washed with cold distilled water, centrifuged | 1.6 | 0.7 |
| Fraction A C-gamma eluate | Alumina C-gamma eluted for 2o min. with 0.2M phosphate buffer, pH 7.0, centrifuged. | 53.6 | 801 |

* $Q_n$ values (see Experimental Results)

Table 7.

pH Optimum for Pyrimidine Nucleoside Phosphorylase

| pH | Percent Maximum Splitting | |
|---|---|---|
| | Experiment 1 | Experiment 2 |
| 6.1 | 65 | -- |
| 6.2 | -- | 87 |
| 6.5 | -- | 88 |
| 6.6 | 68 | -- |
| 6.8 | 96 | -- |
| 7.0 | 95 | -- |
| 7.2 | 100 | 100 |
| 7.5 | 86 | -- |
| 7.6 | -- | 77 |
| 7.9 | -- | 47 |
| 8.0 | 65 | -- |

Experimental conditions: uridine, 0.8 micromoles per ml.; phosphate buffer, 0.03M; enzyme, experiment 1, 0.02 mg. protein-nitrogen per ml., experiment 2, 0.05 mg. protein-nitrogen per ml.; t = 37°C.

Role of phosphate.

With the exception of the yeast nucleosidase of Carter (1951), all nucleosidases studied thus far are phosphorylytic in nature. Phosphate is required for maximum nucleosidase activity; arsenate may be substituted for phosphate in some instances. The necessity for phosphate was borne out in experiments with whole cells and substantiated by the experiments with the purified enzyme.

Figure 8 shows the effect of various concentrations of phosphate ion upon the rate of cleavage of uridine by the enzyme. An enzyme preparation dialyzed free of phosphate was used for the experiments. The data indicate that the rate of splitting is dependent upon the phosphate concentration; only a minimal amount of uridine is cleaved in the absence of phosphate ion.

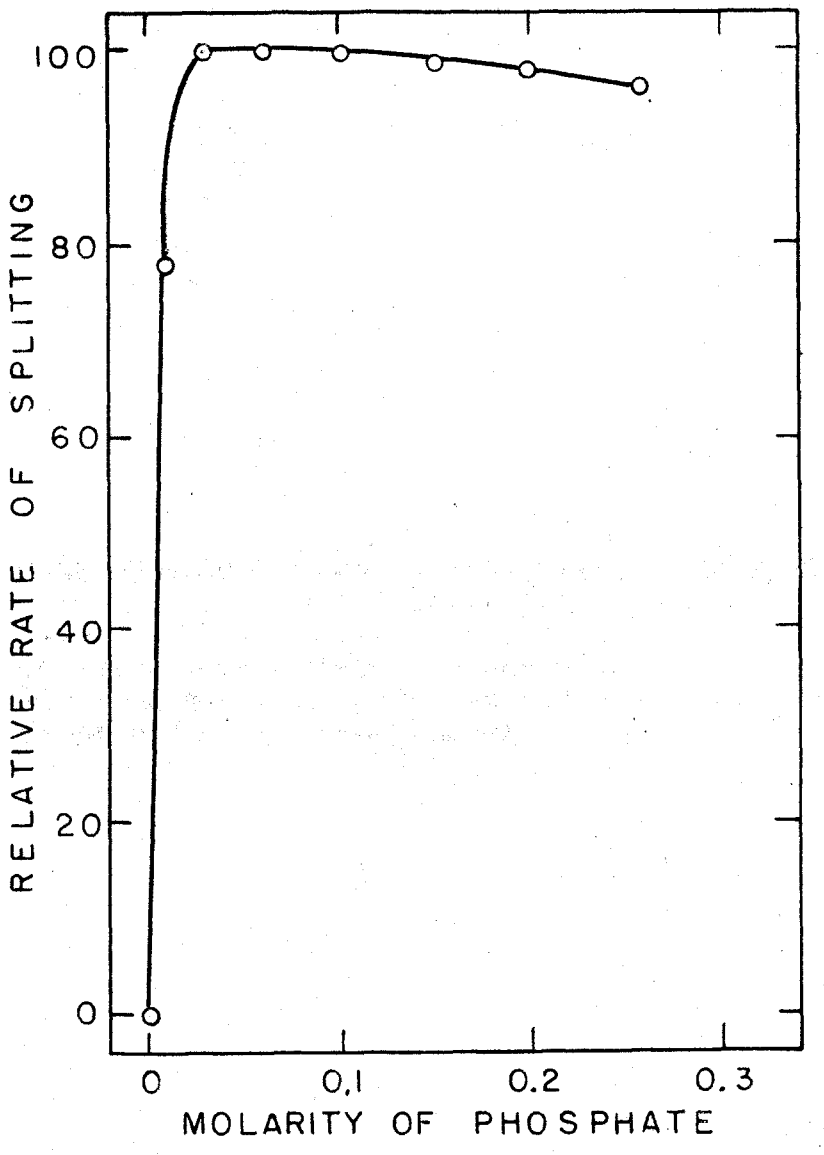
If pyrimidine nucleosidase is a phosphorylytic enzyme, it would be expected to catalyze a reaction similar to that of purine nucleoside phosphorylase. The following reaction would be postulated,

$$\text{Uridine} + \text{phosphate} \rightleftharpoons \text{ribose-1-phosphate} + \text{uracil}$$

The presence of high concentrations of phosphate ion would cause the equilibrium to be shifted to the right and uridine splitting would be favored. Within limits this was shown to

Fig. 8. Effect of Phosphate Concentration on Rate of Splitting.

Experimental conditions: Uridine, 1.01 micromoles per ml.; phosphate buffer, pH 7.0; enzyme, 0.05 mg. protein-nitrogen per ml.; t = 37°C.


RELATIVE RATE OF SPLITTING
100
80
60
40
20
0
0
0.1
0.2
0.3
MOLARITY OF PHOSPHATE

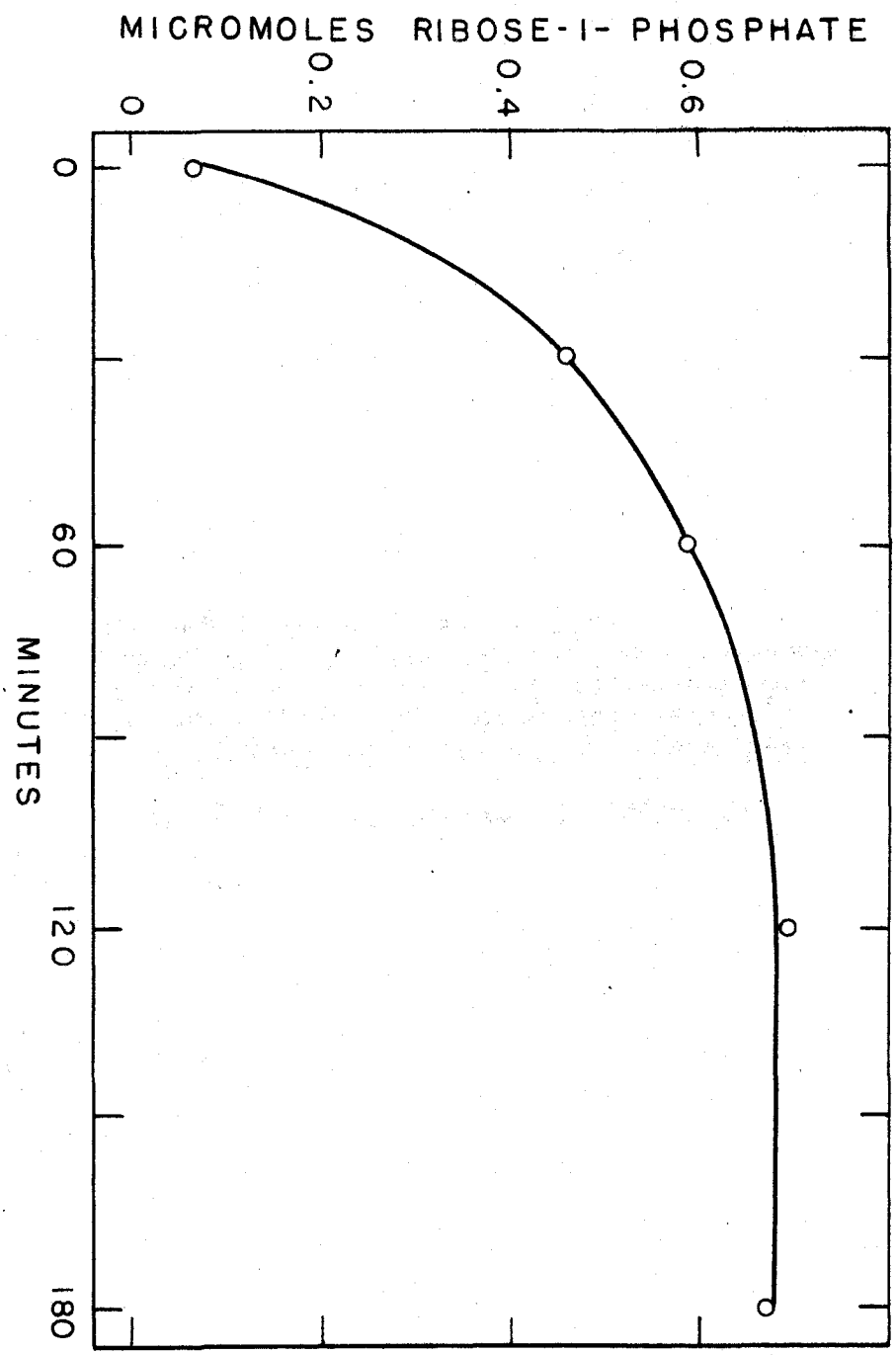
be true. However, concentrations of phosphate ion in excess of 0.1M showed a gradual decline in the amount of uridine split. If the reaction were hydrolytic, the presence of phosphate would exert no effect upon the rate of reaction; indeed, none would be expected since in this case phosphate would not be a part of the equilibrium.

As shown in the reaction above, ribose-1-phosphate would be formed as one of the products if the enzyme were phosphorylytic in nature. Ribose-1-phosphate does accumulate as one of the reaction products. This is illustrated in figure 9. Ribose-1-phosphate is a labile compound but may be determined by the method of Lowry and Lopez (1946) for estimating inorganic phosphorous in the presence of labile phosphoric acid esters. The data obtained for the accumulation of ribose-1-phosphate approximate those for the accumulation of pentose and uracil (Figure 7) described earlier.

Ribose-1-phosphate was also isolated as the barium salt from incubation mixtures in which uridine was metabolized by the enzyme. The product gives no reaction with the test of Lowry and Lopez unless it has been hydrolyzed with dilute mineral acid for 30 min. at 30°C. or for 5 min. in a boiling water bath. Due to its method of formation and its behavior toward hydrolysis with dilute acid, the indication is that the compound is the same as that described by Kalckar (1947c)

Fig. 9. Accumulation of Ribose-1-Phosphate.

Experimental conditions: uridine, 1.01 micromoles per ml.; phosphate buffer, 0.005M, pH 7.0; glycyl-glycine buffer, 0.03M, pH 7.0; enzyme, 0.03 mg. protein-nitrogen per ml.; t = 37°C.


MICROMOLES RIBOSE-1- PHOSPHATE
0
0.2
0.4
0.6
MINUTES
0
60
120
180

as one of the products of the action of purine nucleoside phosphorylase upon purine ribosides. It may thus be stated that like purine nucleoside phosphorylase, the pyrimidine counterpart is also a phosphorylytic enzyme, and according to the nomenclature in use, the enzyme should properly be called pyrimidine nucleoside phosphorylase.

Specificity of pyrimidine nucleoside phosphorylase.

Pyrimidine nucleoside phosphorylase does not metabolize cytidine. This is illustrated in Figure 10. It was indicated earlier that in the case of purine nucleoside phosphorylase, deamination precedes nucleosidase activity (Stephenson and Trim, 1938). This also seems to be the case for pyrimidine nucleosides, since it was shown earlier, that whole cells and extracts of E. coli can deaminate and metabolize cytidine. The enzyme preparation apparently is free of cytidine deaminase, since no appreciable amount of splitting of cytidine occured when it was incubated with the enzyme.

Figure 11 shows the specificity of the enzyme toward purine nucleosides and thymine desoxyriboside. Adenosine and guanosine were not dissimilated by the enzyme; however, inosine was attacked. Crystalline inosine was not available for this experiment; and, inosine was prepared by the

Fig. 10. Specificity of Pyrimidine Nucleoside Phosphorylase. I. Cytidine.

Experimental conditions: cytidine, 1.01 micromoles per ml.; uridine, 1.01 micromoles per ml.; phosphate buffer, 0.04M, pH 7.0; enzyme, 0.02 mg. protein-nitrogen per ml.; t = 37°C. Curve: (△) cytidine; (○) uridine.

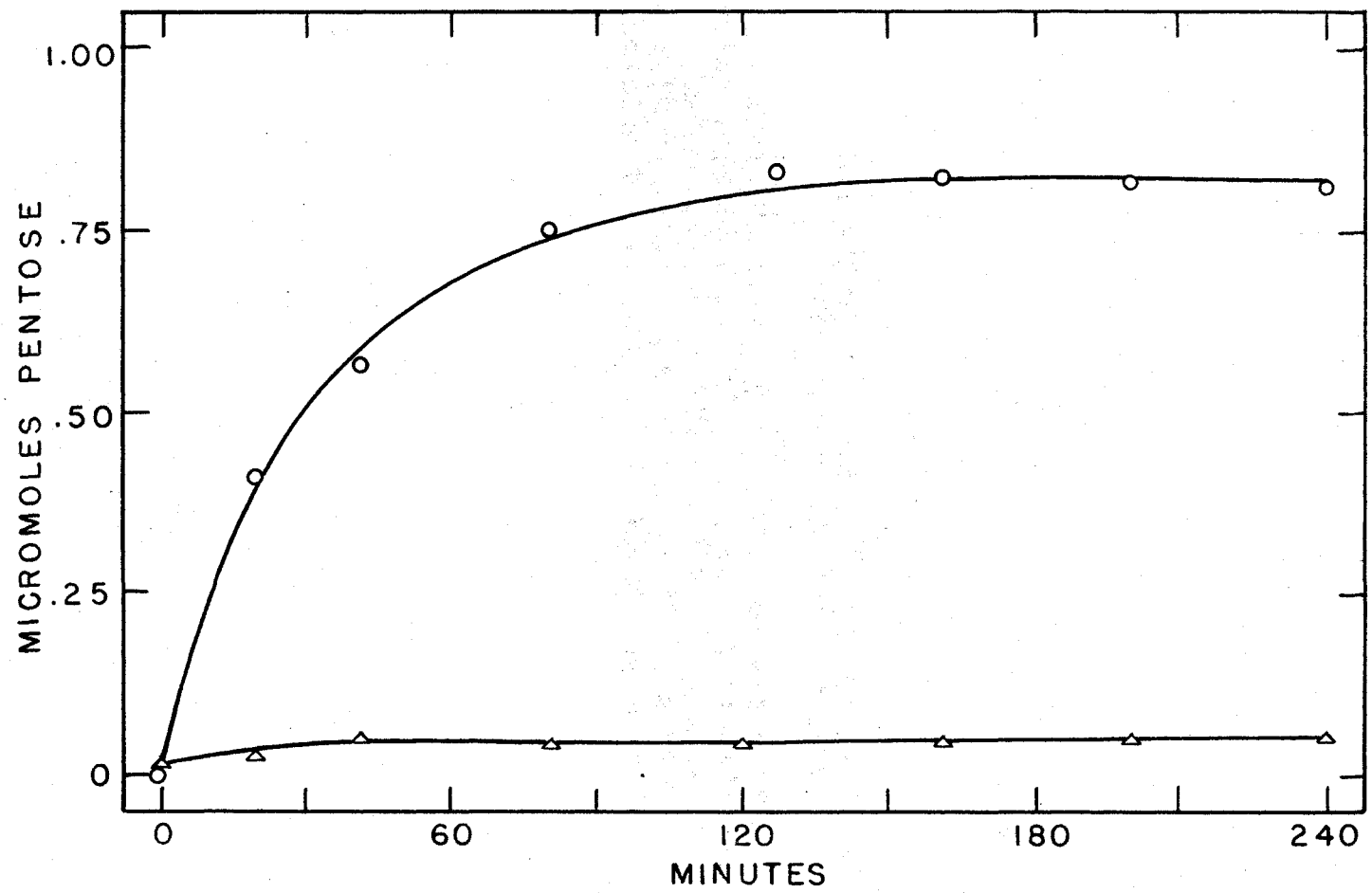
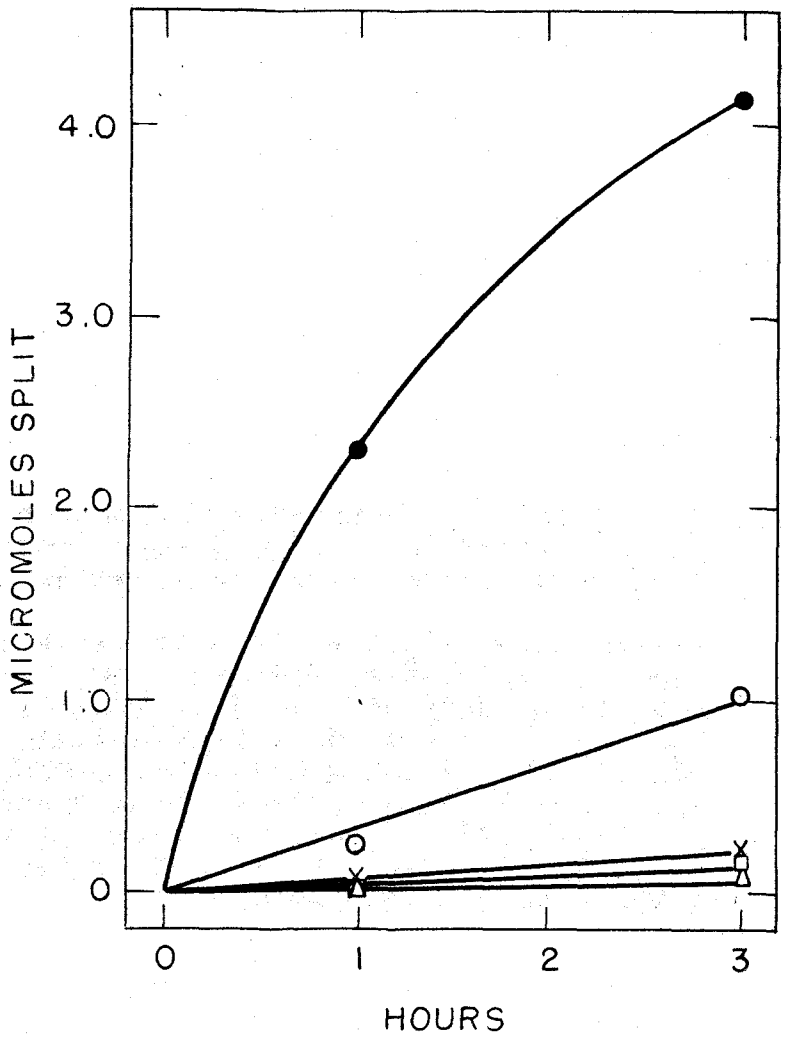

1.00
.75
.50
.25
0
MICROMOLES PENTOSE
0
60
120
180
240
MINUTES

Fig. 11. Specificity of Pyrimidine Nucleoside Phosphorylase. II. Adenosine, Guanosine, Inosine and Thymine Desoxyriboside.

Experimental conditions: uridine, 4.83 mg. per ml.; adenosine, 4.94 mg. per ml.; guanosine, 4.94 mg. per ml.; inosine, 5.0 mg. per ml.; thymine desoxyriboside, 3.54 mg. per ml.; phosphate buffer, 0.05M, pH 7.0; enzyme, 0.08 mg. protein-nitrogen per ml.; t = 37°C. Curve: (•) uridine; (○) inosine; (×) thymine desoxyriboside; (□) guanosine; (△) adenosine.


MICROMOLES SPLIT
4.0
3.0
2.0
1.0
0
0
1
2
3
HOURS

deamination of adenosine with the adenosine deaminase of Brady (1942). The possibility that the deaminase was contaminated with purine nucleoside phosphorylase is highly probable. If such were the case, the results indicated here would not present a true picture of the specifity of the enzyme; the splitting would not be due to pyrimidine nucleoside phosphorylase. It is more likely that inosine is not cleaved by the enzyme, since it failed to attack either adenosine or guanosine. Thymine desoxyriboside was not attacked by the enzyme.

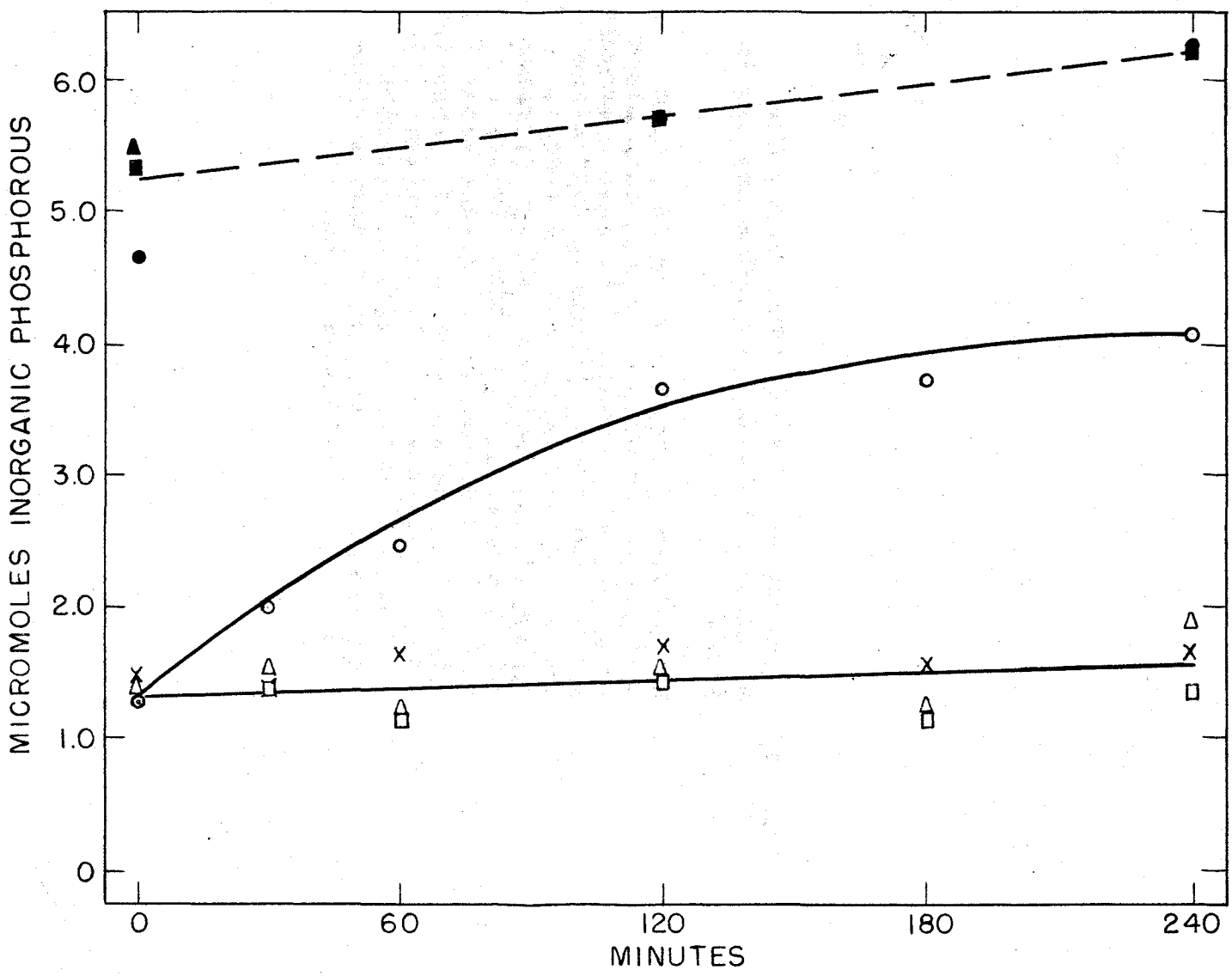
The formation of a riboside could not be demonstrated when either cytosine or orotic acid were incubated with ribose-1-phosphate in the presence of the enzyme. As is illustrated in Figure 12, no increase in inorganic phosphate was observed. This substantiates the statements made with regard to the specificity of the enzyme for cytidine. It also indicates that a nucleosidase other than this pyrimidine nucleoside phosphorylase is operative in the interconversion of orotic acid and orotic acid riboside.

Synthesis of uridine.

Ribose-1-phosphate was isolated as the barium salt from mixtures in which uridine was incubated with pyrimidine nucleoside phosphorylase. The ester was converted to the

Fig. 12. Specificity of Pyrimidine Nucleoside Phosphorylase. III. Cytosine and Orotic Acid.

Experimental conditions: uracil, 4.36 micromoles per ml.; cytosine, 4.42 micromoles per ml.; orotic acid, 4.46 micromoles per ml.; ribose-1-phosphate, 4.37 micromoles per ml.; glycyl-glycine buffer, 0.02M, pH 7.0; enzyme, 0.03 mg. protein-nitrogen per ml.; t = 37°C. Curve: (○) uracil; (□) cytosine; (△) orotic acid; (×) ribose-1-phosphate control; (●) inorganic + labile phosphate, uracil experiment; (■) inorganic + labile phosphate, cytosine experiment; (▲) inorganic + labile phosphate, orotic acid experiment.


MICROMOLES INORGANIC PHOSPHOROUS
6.0
5.0
4.0
3.0
2.0
1.0
0
0
60
120
180
240
MINUTES

sodium salt with sodium sulfate and the barium ion removed as the insoluble barium sulfate.

As was discussed earlier, the presence of phosphate ion favors the cleavage of the uridine molecule. However, in the presence of small amounts of inorganic phosphate, the reaction may be reversed and synthesis of uridine will be favored. This was observed. The enzyme was dialyzed to remove all of the elution buffer. Uracil and ribose-1-phosphate were incubated in the presence of pyrimidine nucleoside phosphorylase. Using the method of Lowry and Lopez (1946), it was demonstrated that inorganic phosphorus increased as the incubation progressed; yet there was no appreciable increase in inorganic phosphate in the control. This indicated that uracil-riboside (uridine) was synthesized, and that phosphoric acid was liberated from the ribose-1-phosphate as it was utilized. The synthesis of uridine is illustrated in Figure 13. The synthesis was also shown spectrophotometrically; the extinction at 290 m decreased due to the disappearance of uracil and the formation of uridine.

Equilibrium of uridine cleavage and synthesis.

The equilibrium of the reaction was studied. Uridine was synthesized according to the procedure described earlier.

Fig. 13. Enzymatic Synthesis of Uridine.

Experimental conditions: uracil, 3.50 micromoles per ml.; ribose-1-phosphate, 3.77 micromoles per ml.; glycyl-glycine buffer, 0.02M, pH 7.0; enzyme, 0.03 mg. protein-nitrogen per ml.; t = 37°C. Curve: (○) uracil; (□) ribose-1-phosphate control; (△) inorganic + labile phophate.

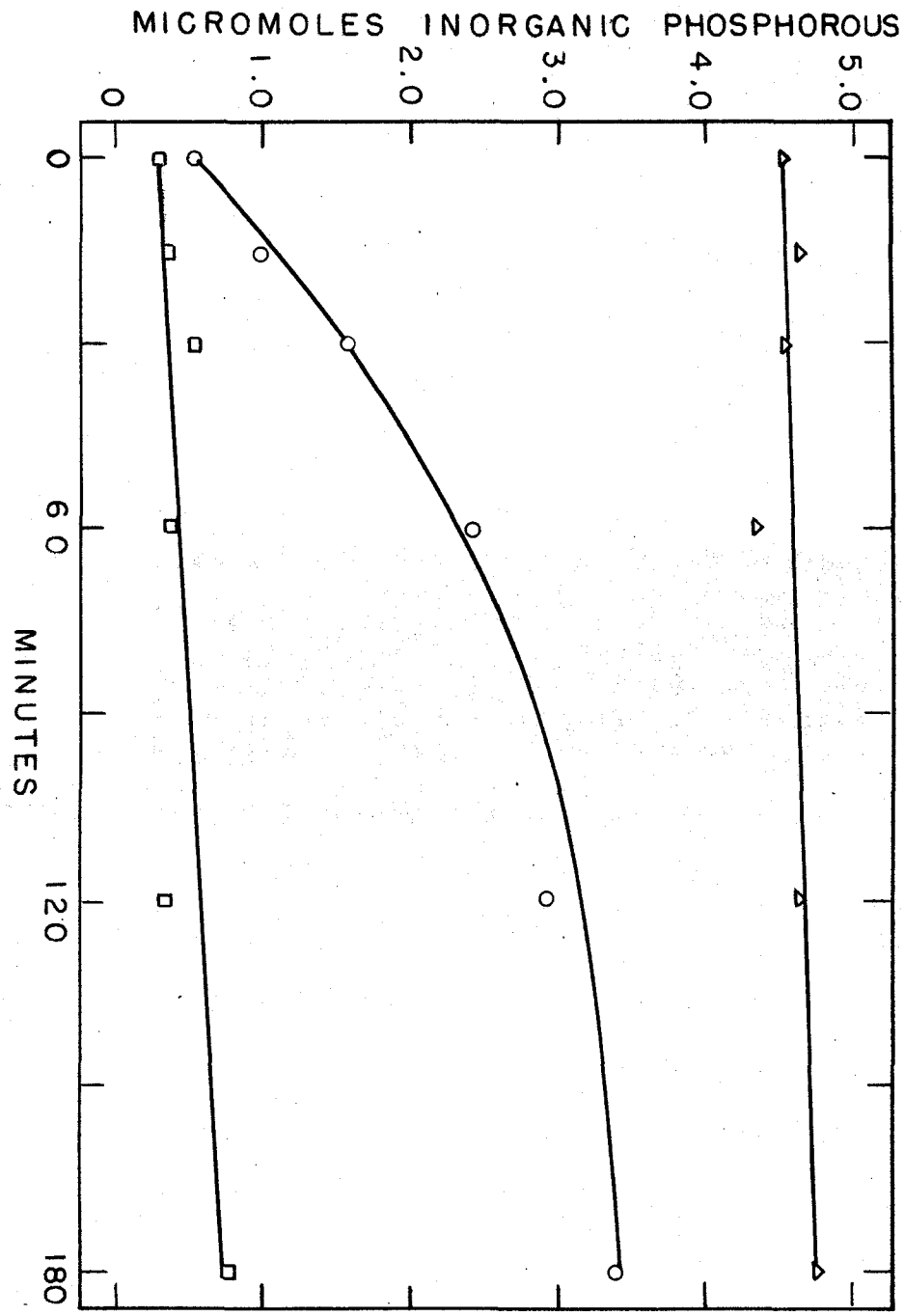
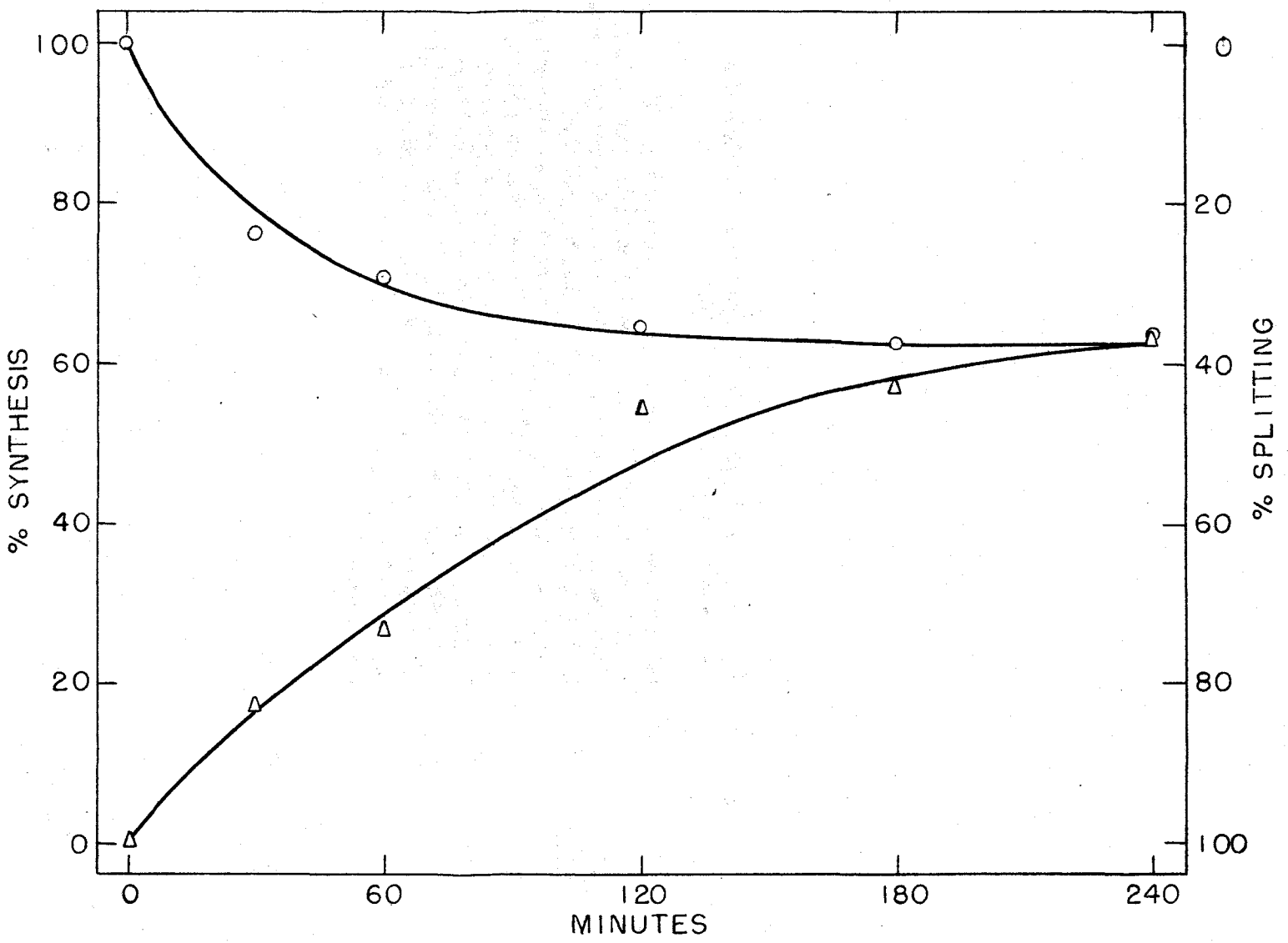

MICROMOLES INORGANIC PHOSPHOROUS
0
1.0
2.0
3.0
4.0
5.0
MINUTES
0
60
120
180

Fig. 14. Equilibrium Between Enzymatic Synthesis and Splitting of Uridine.

Experimental conditions: synthesis of uridine; uracil, 4.36 micromoles per ml.; ribose-1-phosphate, 4.37 micromoles per ml.; glycyl-glycine buffer, 0.02M, pH 7.0; enzyme, 0.03 mg. protein-nitrogen per ml.; t = 37°C. Splitting of uridine; uridine, 4.50 micromoles per ml.; glycyl-glycine buffer, 0.02M, pH 7.0; phosphate buffer, 0.004M, pH 7.0; enzyme, 0.03 mg. protein-nitrogen per ml.; t = 37°C. Curve: (△) synthesis of uridine; (○) splitting of uridine.


100
80
60
40
20
0
% SYNTHESIS
0
20
40
60
80
100
% SPLITTING
0
60
120
180
240
MINUTES

It was observed that the synthesis of uridine approached an equilibrium when approximately 62 percent of the ribose-1-phosphate available was utilized. The equilibrium was also approached from the direction of uridine splitting. Uridine was incubated with inorganic phosphate in the presence of the enzyme. The concentration in inorganic phosphate in the splitting experiment was the same as the final concentration in inorganic phosphorous in the experiment in which uridine was synthesized. In this instance the equilibrium was approached when approximately 38 percent of the uridine introduced was cleaved. The results of these two experiments are shown in Figure 14. It must be emphasized that since the concentration in phosphate ion has such a marked effect upon the position of the equilibrium, the equilibrium established here holds only for the molarity in phosphate indicated; and, a different equilibrium would be established under other phosphate ion concentrations.

# DISCUSSION

The reactions of purine riboside metabolism have long been studied and are quite well known; however, the consideration of pyrimidine riboside metabolism is a rather recent development. The metabolism of pyrimidine ribosides has much in common with the metabolism of purine ribosides. Thus, various investigators were justified in inferring that the dissimilation of pyrimidine compounds occurs in a fashion similar to that of purine compounds. In both instances the ribose split from the nucleoside disappears from the reaction mixture and is rapidly oxidized to carbon dioxide and water. The exact nature of the process, however, still remains obscure. Likewise, in both purine and pyrimidine nucleoside metabolism the free base which is liberated accumulates and may be isolated. Purine and pyrimidine bases are remarkably resistant to structural changes and alteration by enzymes, and are usually recovered intact from reaction mixtures. Only the appendages such as amino, hydroxyl, and oxygen groups undergo change. This is undoubtedly of great importance to organisms, since purine and pyrimidine groups form an integral part of some coenzymes.

The order in which the various enzyme systems attack nucleosides has been studied. With the exception of the

nucleotide-N-ribosidase of Komita (1937, 1938a,b), ribose is not split from nucleotides. The nucleotide must first be dephosphorylated before nucleosidase activity can occur (Stephenson and Trim, 1938). The question now arises as to whether deamination must precede nucleosidase action or whether independent enzyme systems exist for the deamination of nucleosides and the free bases. The literature is conflicting with regard to this point. The investigations of Stephenson and Trim (1938), indicated strongly that deamination of nucleosides must occur before rupture of the base-carbohydrate bond is possible. On the other hand, Wang, et al. (1950) reported that extracts prepared from cells of E. coli deaminated cytidine, cytosine desoxyriboside, adenosine, and guanosine; yet, the same preparation also deaminated cytosine, isocytosine, adenine, and guanine. Recently, Wang and Lampen (1951) indicated that resting cells in the presence of arsenate buffer directly split adenosine to adenine, and cytidine to cytosine without prior deamination. This is in variance with the observations reported here which tend to support the observation of Stephenson and Trim (1938). In the system studied, deamination preceded nucleosidase action. Uridine was actively metabolized by the same enzyme preparation which had no effect upon cytidine (see Figure 10). This indicates

that the pyrimidine nucleoside phosphorylase preparation is free of cytidine deaminase, which if present would have deaminated cytidine to uridine which in turn would undergo dissimilation. It is unlikely that the rate of reaction of cytidine deaminase is so slow that deamination would fail to occur after two hours incubation. The lack of success in the formation of a riboside of cytosine (see Figure 12) also indicates that neither cytidine nor cytosine are components of the equilibrium. Deamination must, therefore, occur before nucleosidase activity can take place.

The first observation that phosphate was necessary for nucleosidase activity came from the experiments of Klein (1935). Somewhat later, Inagaki (1940) found that nucleosidase activity could be shown by gastric and pancreatic juices if inorganic phosphate were provided. The significance of this observation was overlooked, however. Lipmann (1943) reported the possibility of formation of ribose-1-phosphate as a result of nucleosidase action. It was not until 1945 that Kalckar (1945a,b) first isolated the phosphorylated compound of ribose, and proved that purine nucleosidase was phosphorylytic in nature. In agreement with this discovery, it was observed that pyrimidine nucleosidase is also a phosphorlytic enzyme (see Figures 5 and 9). The occurrence of ribose-1-phosphate in reaction mixtures was demonstrated by the method of Lowry and Lopez

(1946) for the determination of inorganic phosphate in the presence of labile organic phosphate esters. This was substantiated by the isolation of the barium salt of ribose-1-phosphate, the reactions of which agree with those shown by Kalckar's ester (1945a,b; 1947c,d). Also, uridine can be synthesized by incubation of uracil with ribose-1-phosphate in the presence of pyrimidine nucleoside phosphorylase. Inorganic phosphate increased as the reaction progressed. There was no phosphatase activity in the experiment since there was no increase in inorganic phosphate in the control, and the inorganic phosphate bound to the ribose was determined at the end of the experiment. Recently, Manson and Lampen (1951) reported that the metabolism of purine and pyrimidine desoxyribosides involved phosphorolysis. The only nucleosidase described thus far that was found non-phosphorolytic is that of Carter (1951). Neither phosphate nor arsenate affected the rate of reaction of this enzyme. It appears that two pathways for the metabolism of nucleosides exist, the phosphorolytic route generally shown and accepted, and an alternative hydrolytic pathway which functions in a rather isolated case.

Pyrimidine nucleoside phosphorylase is a very specific enzyme. Uridine is the only pyrimidine nucleoside attacked by the enzyme. A better name for the enzyme which more completely describes its action would be uridine phos-

phorylase. The enzyme is not active against cytidine (see Figure 10). Neither does it attack purine ribosides (see Figure 11). Adenosine and guanosine are not cleaved by the enzyme, and the splitting of inosine remains doubtful for reasons presented earlier (see page 82). In these respects the enzyme compares with the specificity shown by the pyrimidine nucleosidase of Deutch and Laser (1929). Pyrimidine desoxyribosides are difficult to obtain; only thymine desoxyriboside could be investigated. This is rather unfortunate since it still does not prove that the enzyme is inactive against the desoxyriboside of uridine, which does not occur naturally. Apparently the pyrimidine part of the molecule determines much of the specificity shown by the enzyme. It is presumed that the desoxyriboside of uridine, if available, would be cleaved by the enzyme, since thus far, no specificity for either ribose or desoxyribose has been shown for other nucleosidases.

The importance of nucleosidase activity is illustrated in the incorporation experiments of Plentl and Schoenheimer (1944), Bendich, et al. (1949), Loring and Pierce (1944), and Loring (1944). Pyrimidine nucleosides and nucleotides are incorporated into the pyrimidine fractions of the rat and in the mold Neurospora; however, pyrimidine bases do not serve as precursors of nucleoprotein. The lack of incorporation of the free bases suggests that either the rate of

synthesis of nucleosides is involved, or the rate of synthesis of ribose-1-phosphate is the limiting factor. In either case, nucleosidase activity might be the critical element, since it is involved in the synthesis of ribose-1-phosphate through the splitting of existing nucleosides; and, it is also involved as the enzyme catalyzing the attachment of the base and ribose-1-phosphate. When the equilibrium that exists between synthesis and splitting of pyrimidine nucleosides is considered (see Figure 14), the reason for the lack of incorporation of pyrimidine bases can be postulated. At the phosphate concentration employed in the experiments, the equilibrium was approximately sixty percent in favor of synthesis and but forty percent in favor of splitting. As the phosphate concentration is increased, the equilibrium is shifted in the direction of cleavage; and, as the phosphate concentration is decreased the equilibrium is directed toward synthesis. The phosphate concentration of the experiment described, is much higher than the phosphate concentration of the body fluids; thus in the animal organism the tendency toward synthesis over splitting is even greater. Since ribose-1-phosphate arises from cleavage of nucleosides, the suggestion that it is the critical factor restricting the incorporation of pyrimidine bases appears likely.

The role of orotic acid in the metabolism of pyrimidine nucleosides has been of special interest since Mitchell, et al. (1948) observed that pyrimidine-requiring mutants of Neurospora accumulated large amounts of the substance. Two schemes for the incorporation of orotic acid appear possible.

(1) Orotic acid $\xrightarrow{-CO_2}$ uracil $\rightarrow$ uridine

(2) Orotic acid $\rightarrow$ orotic acid riboside $\xrightarrow{-CO_2}$ uridine

Attempts to demonstrate the direct decarboxylation of orotic acid were unsuccessful (see page 60). This seems to eliminate scheme (1). The experiments of Hammarsten and co-workers (Arvidson, et al., 1949; Bergstrom, et al., 1949; Reichard, 1949; Hammarsten and Reichard, 1950) and those of Weed, et al. (1950) indicated the rapid incorporation of orotic acid into the pyrimidine nucleotide fraction of ribonucleic acid. On the basis of these investigations, the formation of an orotic acid riboside according to scheme (2) was expected. This could not be shown (see p. 60; Figure 12). The reaction,

Pyrimidine riboside + phosphate $\rightleftharpoons$
pyrimidine base + ribose-1-phosphate

could not be reversed through the presence of orotic acid. This would suggest that an enzyme separate and distinct from pyrimidine nucleoside phosphorylase catalyzes the system, or that orotic acid is not utilized in this fashion. There

is no doubt that the first postulate is true; the enzyme exhibits no action on orotic acid or vice versa. That orotic acid is utilized by the pathway outlined in scheme (2) was demonstrated by Michelson, et al. (1951). These workers were able to isolate a new nucleoside, "orotidine" (orotic acid riboside) from yeast. The substance is sensitive to hydrolysis by heat in the presence of a mineral acid. This would explain the failure to obtain positive results in experiments in which the Mejbaum pentose test (1939) was used. The failure of the enzyme described here, to combine orotic acid with ribose-1-phosphate, however, is demonstrated in a reliable way by phosphate analyses. Any reaction would have resulted in the mineralization of the labile organic phosphate ester.

The present status of pyrimidine nucleosidases is as follows. Separate enzymes exist for thymidine and uridine. Desoxycytidine and cytidine may possibly be split by the same enzyme; since no separate purification of the enzymes has been accomplished so far. The existence of a separate orotidine nucleosidase is certain. The non-phosphorylating pyrimidine nucleosidase of yeast acts apparently by splitting the nucleoside irreversibly. This causes difficulty in explaining the formation of nucleosides. With the current investigation, rigorous proof of phosphorolytic

action of at least one of the pyrimidine nucleosidases has been furnished. The purification of all other enzymes of this group has not been accomplished thus far, and all data on their specificity and mode of action must necessarily be taken with caution.

## SUMMARY AND CONCLUSIONS

1. The metabolism of pyrimidine nucleosides was found to be similar to the dissimilation of purine nucleosides. The pyrimidine base is cleaved from the nucleoside and is accumulated. Ribose disappears from the reaction mixture; it is rapidly oxidized to carbon dioxide and water.

2. Pyrimidine-bound ribose cannot be determined by the orcinol test of Mejbaum (1939). A modified orcinol reaction for this purpose was developed and used in the study of pyrimidine riboside metabolism.

3. Pyrimidine nucleoside phosphorylase was isolated from extracts of E. coli and brought to a high degree of purity by fractional precipitation with ammonium sulfate and adsorption on and elution from alumina C-gamma. The purified enzyme shows a six-hundred-fold increase in activity over that of the cell-free extract from which it was prepared.

4. Pyrimidine nucleoside phosphorylase is a phosphorolytic enzyme. Inorganic phosphate is necessary for its action. Ribose-1-phosphate is accumulated as a result of its reaction upon nucleosides. The presence of ribose-1-phosphate was determined by analytical methods and by isolation as the barium salt.

5. The enzyme is highly specific. It does not attack cytidine. It is inactive against purine nucleosides. It will not split thymine desoxyriboside. Cytosine, thymine, and orotic acid do not react with ribose-1-phosphate in the presence of the enzyme. The role of orotic acid in the anabolism of pyrimidine nucleosides is discussed.

6. Uridine was synthesized. Uracil when incubated with ribose-1-phosphate in the presence of pyrimidine nucleoside phosphorylase was converted to uridine. The equilibrium between uridine synthesis and splitting was determined.

## LITERATURE CITED

Arvidson, H., Eliasson, N. A., Hammarsten, E., Reichard, P., Ubisch, H. v., and Bergström, S.
1949. Orotic acid as a precursor of pyrimidines in the rat. J. Biol. Chem. 179, 169-173.

Bagatell, F. K., Ames, Iowa. Utilization of pyrimidine ribosides by Aerobacter aerogenes. (Private communication.) 1951.

Barker, G. R.
1950. The determination of carbohydrate residues in pentose nucleic acids. J. Chem. Soc. 1950, 1636-1637.

Bendich, A., Getler, H. and Brown, G. B.
1949. A synthesis of isotopic cytosine and a study of its metabolism in the rat. J. Biol. Chem. 177, 565-570.

Bergström, S., Arvidson, H., Hammarsten, E., Eliasson, N., Reichard, P., and Ubisch, H. v.
1949. Orotic acid, a precursor of pyrimidines in the rat. J. Biol. Chem. 177, 495.

Brady, T.
1942. Adenosine deaminase. Biochem. J. 36, 3478-3484.

Bredereck, H., Martini, A. and Richter, F.
1941. Fermentative und chemische Darstellung der Nucleotide aus Hefenucleinsäure. Ber. chem. Ges. 74, 694-697.

Caputto, R., Leloir, L. F., Cardini, C. E. and Paladini, A. C.
1950. Isolation of the coenzyme of galactose phosphate-glucose phosphate transformation. J. Biol. Chem. 184, 333-350.

Cardini, C. E., Paladini, A. C., Caputto, R. and Leloir, L. F.
1950. Uridine-diphosphate glucose: the enzyme of the galactose-glucose phosphate isomerization. Nature 165, 191-192.

_____, _____, _____, and _____.
1950. Liver uridine phosphorylase. Acta physiol. Latinoamericana 1, 57-63. Original article not available. Abstract in C. A. 45, 1173i, 1951.

Carter, C. E.
1951. Partial purification of a non-phosphorylytic uridine nucleosidase from yeast. J. Am. Chem. Soc. 73, 1508-1510.

Colowick, S. P.
1947. Eli Lilly award in biochemistry. Hexokinade and phosphofructokinase. Paper presented at the 112th. Meeting Am. Chem. Soc., New York, 56c-57c.

_____ and Price, W. H.
1945. Guanine as a coenzyme in enzymatic transphosphorylation. J. Biol. Chem. 159, 563-564.

_____ and _____.
1946. Enzymic formation of guanine by a reversible phosphorolytic cleavage of ribonucleic acid. Federation Proc. 5, 130.

Cori, C. F., Cori, G. T., and Green, A. A.
1943. Crystalline muscle phosphorylase. III. Kinetics. J. Biol. Chem. 151, 39-55.

Deutsch, W. and Laser, R.
1929. Experimentelle Studien über den Nucleinstoffwechsel. XIX. Mitteilung. Zur Kenntnis der Nucleosidase.-Verhalten einer Nucleosidase aus Rinderknochenmark zu einem Spaltprodukt der Thymusnucleinsäure. Z. physiol. Chem. 186, 1-10.

Dixon, M. and Lemberg, R.
1934. Studies on xanthine oxidase. XII. The oxidation of nucleosides. Biochem. J. 28, 2065-2079.

Dmochowski, A., Zajdenman, A. and Rabanowska, A.
1935. Über die jodometrische Bestimmung des Muskelguanins. Biochem. Z. 279, 289-295.

Doudoroff, M.
1943. Studies on the phosphorolysis of sucrose. J. Biol. Chem. 151, 351-361.

Dunstan, S. and Gillam, A. E.
1949. A micro-method for the determination of pentoses by photoelectric spectrophotometry. J. Chem. Soc. 1949, S140-S144.

Euler, H. v. and Brunius, E.
1927. Zur Kenntnis der Nucleosidasen. Ber. 60B, 1584-1591.

Fiske, C. H. and SubbaRow, Y.
1925. The colorimetric determination of phosphorous. J. Biol. Chem. 66, 375-400.

Friedkin, M.
1950. Desoxyribose-1-phosphate. II. The isolation of crystalline desoxyribose-1-phosphate. J. Biol. Chem. 184, 449-459.

_____ and Kalckar, H. M.
1950. Desoxyribose-1-phosphate. I. The phosphorolysis and resynthesis of purine desoxyribose nucleoside. J. Biol. Chem. 184, 437-447.

_____, Kalckar, H. M. and Hoff-Jørgensen, E.
1949. Enzymatic synthesis of desoxyribose nucleoside with desoxyribose phosphate ester. J. Biol. Chem. 178, 527-528.

Grynberg, M. Z.
1932. Eine jodometrische Methode zur Bestimmung von einigen Purinen und ihren Abkömmlingen. Biochem. Z. 253, 143-145.

Hammarsten, E. and Reichard, P.
1950. Pyrimidine nucleosides as precursors of pyrimidines in polynucleotides. J. Biol. Chem. 183, 105-109.

Hoff-Jørgensen, E., Friedkin, M., and Kalckar, H. M.
1950. Desoxyribose-1-phosphate. III. Comparison of microbiological and spectrophotometric estimations of enzymatically produced desoxyribose nucleoside. J. Biol. Chem. 184, 461-464.

Hotchkiss, R. D.
1948. The quantitative separation of purines, pyrimidines and nucleosides by paper chromatography. J. Biol. Chem. 175, 315-332.

Inagaki, T.
1940. Studies on hydrolysis of desoxyribonucleotides and nucleosides. J. Biochem. (Japan) 32, 63-79.

Ishikawa, H. and Komita, Y.
1936. Enzymic hydrolysis of nucleotides and nucleosides. I. Hydrolysis of purine ribonucleotides. J. Biochem. (Japan) 23, 351-363.

Iwanoff, L.
1903. Ueber die fermentative Zersetzung der Thymonucleinsäure durch Schimmelpilze. Z. physiol. Chem. 39, 31-43.

Jones, W.
1911a. Concerning nucleases. J. Biol. Chem. 9, 129-137.

Jones, W.
1911b. On the physiological agents which are concerned in the nuclein fermentation, with special reference to four independent desaminases. J. Biol. Chem. 9, 169-180.

Jones, W.
1920. Nucleic acids, their chemical properties and physiological conduct. Longmans, Green and Co., London.

Kalckar, H. M.
1945a. Enzymatic synthesis of a nucleoside. J. Biol. Chem. 158, 723-724.

_____.
1945b. Enzymatic synthesis of nucleosides. Federation Proc. 4, 248-252.

_____.
1945c. Enzymatic microanalysis of purine compounds. J. Biol. Chem. 158, 313-314.

Kalckar, H. M.
1947a. Differential spectrophotometry of purine compounds by means of specific enzymes. I. Determination of hydroxypurine compounds. J. Biol. Chem. 167, 429-443.

_____.
1947b. Differential spectrophotometry of purine compounds by means of specific enzymes. II. Determination of adenine compounds. J. Biol. Chem. 167, 445-459.

_____.
1947c. Differential spectrophotometry of purine compounds by means of specific enzymes. III. Studies of the enzymes of purine metabolism. J. Biol. Chem. 167, 461-475.

_____.
1947d. The enzymatic synthesis of purine ribosides. J. Biol. Chem. 167, 477-486.

Kalnitsky, G., Utter, M. F. and Werkman, C. H.
1945. Active enzyme preparations from bacteria. J. Bact. 49, 595-602.

Klein, W.
1935. Experimentelle Studien über den Nucleinstoffwechsel. XXXVII. Über Nucleosidase. Z. physiol. Chem. 231, 125-148.

Komita, Y.
1937. Studies on "Nucleotide-N-ribosidase". J. Biochem. (Japan) 25, 405-416.

_____.
1938a. Enzymic hydrolysis of nucleotides and nucleosides. II. Further purification of nucleotide-N-ribosidase. J. Biochem. (Japan) 27, 23-33.

_____.
1938b. Enzymic hydrolysis of nucleotides and nucleosides. III. Hydrolysis of nucleotides and nucleosides by digestive juices and tissue extracts. J. Biochem. (Japan) 27, 191-198.

Levene, P. A. and Bass, L. W.
1931. Nucleic acids. The Chemical Catalog Co., Inc., New York.

_____ and Dmochowski, A.
1931. The comparative rates of hydrolysis of adenylic, guanylic, and xanthylic acids. J. Biol. Chem. 93, 563-570.

_____, Jacobs, W. A. and Medigreceanu, F.
1912. On the action of tissue extracts containing nucleosidase on α and β methylpentosides. J. Biol. Chem. 11, 371-380.

Levene, P. A. and LaForge, F. B.
1910. Über die Hefe-Nucleinsäure, III. Ber. chem. Ges. 43, 3150-3163.

_____ and _____.
1912. Über die Hefe-Nucleinsäure. V. Die Struktur der Pyrimidin-Nucleoside. Ber. chem. Ges. 45, 608-620.

_____ and _____.
1913. On nucleases. J. Biol. Chem. 13, 507-509.

_____ and Medigreceanu, F.
1911a. On nucleases. J. Biol. Chem. 9, 65-83.

_____ and _____.
1911b. The action of gastrointestinal juices on nucleic acids. J. Biol. Chem. 9, 375-388.

_____ and _____.
1911c. On nucleases. J. Biol. Chem. 9, 389-402.

_____ and Weber, I.
1924a. On nucleosidases. II. Purification of enzyme. J. Biol. Chem. 60, 707-715.

_____ and _____.
1924b. On nucleosidases. III. The degree of specificity of nucleosidase and the distribution of it in various organs and in various species. J. Biol. Chem. 60, 717-720.

_____, Yamagawa, M. and Weber, I.
1924. On nucleosidases. I. General properties. J. Biol. Chem. 60, 693-706.

Lipmann, F.
1943. Biological oxidation and reductions. Ann. Rev. Biochem. 12, 1-26.

Loring, H. S.
1944. Biochemistry of the nucleic acids, purines and pyrimidines. Ann. Rev. Biochem. 13, 295-314.

_____ and Pierce, J. G.
1944. Pyrimidine nucleosides and nucleotides as growth factors for mutant strains of Neurospora. J. Biol. Chem. 153, 61-69.

Lowry, O. H. and Lopez, J. A.
1946. The determination of inorganic phosphate in the presence of labile phosphate esters. J. Biol. Chem. 162, 421-428.

Lutwak-Mann, C.
1936. The decomposition of adenine compounds by bacteria. Biochem. J. 30, 1405-1412.

MacLeod, M. and Robison, R.
1929. The application of the iodometric method to the estimation of small amounts of aldoses. Biochem. J. 23, 517-523.

Manson, L. A. and Lampen, J. O.
1947. Metabolism of desoxyribosides in Escherichia coli. Federation Proc. 9, 397.

Manson, L. A. and Lampen, J. O.
1951a. Some chemical properties of desoxyribose nucleosides. J. Biol. Chem. 191, 87-93.

_____ and _____.
1951b. The metabolism of hypoxanthine desoxyriboside in animal tissues. J. Biol. Chem. 191, 95-104.

Massart, L. and Hoste, J.
1947. Le dosage photometrique des nucleosides puriques et pyrimidiques au moyen de la reaction a l'orcine. Biochim. Biophys. Acta 1, 83-86.

Mejbaum, W.
1939. Über die Bestimmung kleiner Pentose Mengen, inbesondere in Derivation der Adenylsäure. Z. physiol. Chem. 258, 117-120.

Michelson, A. M., Drell, W. and Mitchell, H. K.
1951. A new ribose nucleoside from Neurospora: "Orotidine". Proc. Natl. Acad. Sci. 37, 396-399.

Mitchell, H. K., Houlahan, M. B. and Nyc, J. F.
1948. The accumulation of orotic acid by a pyrimidine-less mutant of Neurospora. J. Biol. Chem. 172, 525-529.

Mitchell, H. K. and Nyc, J. F.
1947. Intermediates in the synthesis of orotic acid from oxalacetic acid and urea. J. Am. Chem. Soc. 69, 674-677.

Müller, R.
1897. Einwirkung von Oxalessigester auf Guanidin und Harnstoffabkömmlinge. J. prakt. Chem. (2)56, 475-506.

Pierce, W. C. and Haenisch, E. L.
1940. Quantitative analysis. John Wiley and Sons, New York.

Plentl, A. A. and Schoenheimer, R.
1944. Studies in the metabolism of purines and pyrimidines by means of isotopic nitrogen. J. Biol. Chem. 153, 203-217.

Ploeser, J. McT. and Loring, H. S.
1949. The ultraviolet absorption spectra of the pyrimidine ribonucleosides and ribonucleotides. J. Biol. Chem. 178, 431-437.

Reichard, P.
1949. On the turnover of purines and pyrimidines from polynucleotides in the rat determined with $N^{15}$. Acta Chem. Scand. 3, 422-432.

Russell, J. A.
1944. The colorimetric estimation of small amounts of ammonia by the phenol hypochlorite reaction. J. Biol Chem. 156, 457-461.

Schaedel, M. L., Waldvogel, M. J., and Schlenk, F.
1947. The specificity of adenosine deaminase and purine nucleosidase. J. Biol. Chem. 171, 135-141.

Schlenk, F.
1949. Chemistry and enzymology of nucleic acids. Advances in Enzymol. 9, 455-535.

_____ and Waldvogel, M. J.
1946. Note on the metabolism of some ribose derivatives. Arch. Biochem. 9, 455-456.

_____ and _____.
1947. Studies on the metabolism of some ribose derivatives. Arch. Biochem. 12, 181-190.

Schmidt, G. „
1932. Über den fermentativen Abbau der Guanylsäure in der Kaninchenleber. Z. physiol. Chem. 208, 185-224.

Stephenson, M. and Trim, A. R.
1938. The metabolism of adenine compounds by Bact. coli. Biochem. J. 32, 1740-1751.

Vischer, E. and Chargaff, E.
1948. The separation and quantitative estimation of purines and pyrimidines in small amounts. J. Biol. Chem. 176, 703-714.

Wang, T. P.
1950. New nucleosidases in Lactobacillus pentosus. Federation Proc. 9, 399.

_____ and Lampen, J. O.
1951. The cleavage of adenosine, cytidine, and xanthosine by Lactobacillus pentosus. J. Biol. Chem. 192, 339-347.

_____, Sable, H. Z. and Lampen, J. O.
1950. Enzymatic deamination of cytosine nucleosides. J. Biol. Chem. 184, 17-28.

Weed, L. L., Edmonds, M. and Wilson, D. W.
1950. Conversion of radioactive orotic acid into pyrimidine nucleotides of nucleic acid by slices of rat liver. Proc. Soc. Exptl. Biol. Med. 75, 192-

Weichselbaum, T. E.
1946. An accurate and rapid method for the determination of proteins in small amounts of blood serum and plasma. Am. J. Clin. Path. 7, 40-49.

Willstatter, R. and Kraut, H.
1923. Zur Kenntis der Tonerdehydrate. (I. Mitteilung über Hydrate und Hydrogele). Ber. chem. Ges. 56, 149-162.

Willstatter, R. and Schudel, G.
1918. Bestimmung von Traubenzucker mit Hypojodit.
Ber. chem. Ges. 51, 780-781.

ACKNOWLEDGEMENT

The author wishes to express his sincere appreciation to Dr. F. Schlenk for his helpful suggestions and guidance during this work. He also wishes to thank Dr. C. H. Werkman, head of the department, for his cooperation.